OXYGEN SUPPLY AND DEMAND IN HUMANS: CARDIOVASCULAR AND METABOLIC IMPLICATIONS

by

Jayson Reed Gifford

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STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Jayson Reed Gifford**

has been approved by the following supervisory committee members:

Russell S. Richardson	, Chair	**05-06-2015** Date Approved
David Walter Wray	, Member	**05-06-2015** Date Approved
John David Symons	, Member	**05-06-2015** Date Approved
Robert Payne	, Member	**05-06-2015** Date Approved
Flemming Dela	, Member	**05-06-2015** Date Approved

and by **Janet Shaw** , Chair/Dean of

the Department/College/School of **Exercise and Sport Science**

and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

The purpose of this dissertation was to elucidate the regulation of skeletal muscle oxygen (O_2) supply and demand and determine their influence on physical function in health and disease. In the first study, motivated by the theory that heat generated by a muscle attunes O_2 supply to metabolic demand, we explored the mechanisms by which moderate heat inhibits α-adrenergic vasocontraction in isolated human skeletal muscle feed arteries. Of note, the previously recognized sympathoinhibitory effect of heat on α_1-adrenergic vasocontraction could be prevented by inhibiting the temperature-sensitive TRPV4 ion channels or by endothelial denudation, which indicates that TRPV4 ion channels contribute to this response in an endothelium-dependent manner. In the second study we sought to determine if qualitative changes in mitochondrial O_2 consumption contribute to the exercise intolerance exhibited by patients with chronic obstructive pulmonary disease (COPD). Compared to that of healthy controls, permeabilized muscle fibers from the vastus lateralis of patients with COPD exhibited a reduced mitochondrial respiratory capacity that was related to a less-efficient pattern of respiration. Importantly, this altered pattern of respiration, which likely demands more O_2 to resynthesize adenosine triphosphate (ATP), was correlated with knee extensor (KE) endurance among patients, suggesting that altered mitochondrial O_2 demand contributes to exercise intolerance in COPD. In the third study, inspired by the theory of symmorphosis, which postulates that no single step of the O_2 cascade restricts maximal skeletal muscle O_2 consumption (VO_{2max}), we investigated the role of O_2 supply and demand in determining

VO_{2max} in endurance exercise-trained and untrained humans by comparing *in vivo* (skeletal muscle VO_{2max}, direct Fick) and *in vitro* (permeabilized muscle fiber mitochondrial VO_{2max}) measures of respiratory capacity. Interestingly, skeletal muscle VO_{2max} of untrained subjects was limited by mitochondrial O_2 demand, while in trained subjects, who exhibited a training-induced mitochondrial reserve, VO_{2max} was limited by O_2 supply. These findings challenge the concept of symmorphosis by clearly revealing unique constraints to VO_{2max} in untrained and trained humans. In summary, this set of studies helps to elucidate the mechanisms that regulate O_2 supply and demand, and clarifies the influence of these factors on physical function in health and disease.

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CHAPTER 1

INTRODUCTION

In a coordinated chain of events, known as the O_2 cascade, multiple systems and processes function together to deliver atmospheric O_2 to the mitochondria of skeletal muscle and other organs to facilitate ATP resynthesis through oxidative phosphorylation. The O_2 cascade begins with the lungs, which take in air allowing the diffusion of ambient O_2 through the alveoli into the blood. The cardiovascular system then directs the O_2-rich blood to the organs. In the skeletal muscle, the O_2 diffuses through the sarcolemma into the cells, ultimately reaching its destination, the mitochondria, where it participates in oxidative phosphorylation (Rowell, 1992; Richardson, 1998). For simplicity, the steps of the O_2 cascade can be generalized into two components: O_2 supply to the mitochondria and O_2 demand or consumption by the mitochondria.

While much is understood about the O_2 cascade, the mechanisms behind certain processes and implications of alterations in O_2 supply and demand remain uncertain. Therefore, the purpose of this dissertation was to examine three separate, yet related, topics regarding O_2 supply and demand during exercise in health and disease. The first study investigated how the cardiovascular system supplies O_2-rich blood to the active muscle during exercise. The second study examined the influence of alterations in skeletal muscle mitochondrial O_2 consumption on exercise tolerance in patients with COPD. Finally, the third study investigated whether O_2 supply or O_2 demand limit VO_{2max} in untrained and endurance exercise-trained individuals.

The Role of the Cardiovascular System in Supplying O_2-Rich Blood to Active Muscle

At the onset of dynamic exercise there is a dramatic increase in demand for O_2 by the exercising muscle. To meet this demand, the cardiovascular system must increase blood flow to the exercising muscle from 5 to >80% of maximum cardiac output (Rowell, 1992; Laughlin, 1999). As there is a finite cardiac output, this large demand for blood flow can only be achieved by diverting blood away from other organs. Therefore, at the onset of exercise, acting through both feed-forward (*i.e.*, central command) and rapid feedback mechanisms (*e.g.*, baroreceptors), the cardiovascular system initiates a systemic sympathetically-mediated vasoconstriction response to redirect the limited blood supply away from inactive organs and towards the active muscle where perfusion is now needed (Fadel, 2013; Mitchell, 2013). As the sympathetic vasoconstrictor response is systemic in nature, even the vasculature feeding the exercising muscle receives the signal to vasoconstrict, which if left without opposition would result in decreased blood flow and O_2 delivery to the active muscle. In healthy subjects this systemic vasoconstrictor response is met with opposition within the active muscle which, a phenomenon known as functional sympatholysis (Remensnyder *et al.*, 1962), facilitates localized vasodilation in the face of the systemic vasoconstrictor response.

As impaired functional sympatholysis can result in malperfusion, insufficient O_2 supply to the muscle and exercise intolerance, uncovering the factors that mediate sympatholysis has been the subject of many investigations (Hansen *et al.*, 2000; Kirby *et al.*, 2008; Mortensen *et al.*, 2012; Saltin & Mortensen, 2012). At this point it appears that byproducts of the metabolism occurring in the muscle are largely responsible for this

response and are thought to elicit vasodilation by stimulating smooth muscle hyperpolarization and nitric oxide and/or prostanoid release (Hansen *et al.*, 2000; Sarelius & Pohl, 2010). However, which metabolic byproducts are responsible for initiating sympatholysis is still unclear. Recently, considerable attention has been focused upon adenosine and ATP found within the muscle interstitial fluid as their presence has been reported to attenuate sympathetically-mediated vasoconstriction. (Mortensen *et al.*, 2009a; Mortensen *et al.*, 2009b). Other putative metabolic effectors of functional sympatholysis include K^+, H^+, and radiating heat (Hansen *et al.*, 2000; Sarelius & Pohl, 2010).

As one of the most abundant byproducts of relatively inefficient mitochondrial metabolism, heat has attracted significant attention as a potential mediator of sympatholysis. *In vitro* experiments using isolated arteries have been useful in investigating the effect of heat on vascular function (Kluess *et al.*, 2005; Ives *et al.*, 2011; Ives *et al.*, 2012a). Using isolated human skeletal muscle feed arteries, Ives *et a*l. (2011; 2012a) reported that the vasocontractile response to the α_1 adrenergic agonist, phenylephrine (PE), was attenuated in an NO-dependent manner when the arteries were heated to temperatures similar to those experienced by the muscle during exercise (39°C). While it is clear that the sympatholytic effect observed by Ives *et al.* (2011; 2012a) was mediated by endothelial nitric oxide synthase (eNOS) (Ives *et al.*, 2012a), it is unclear what actually senses the heat and initiates this response. **Thus, the first study of this dissertation examined the mechanisms involved in sensing and mediating this sympatholytic effect of heat in isolated human skeletal muscle feed arteries.**

Skeletal Muscle Mitochondrial Respiratory Function and O_2 Demand in Exercise

Mitochondria, ubiquitous organelles, act as the terminal consumer of the O_2 supplied by the cardiopulmonary system in the O_2 cascade. These organelles, which weave throughout a cell in the form of a reticulum, serve many important functions, the most notable of these functions being ATP resynthesis via oxidative phosphorylation (Hatefi, 1985). In the process of oxidative phosphorylation, O_2 reduction (*i.e.*, O_2 consumption) is coupled with the phosphorylation of ADP to form ATP. In this process, illustrated in Figure 1.1, hydrogen ions are funneled down a chemiosmotic gradient from the intermembrane space across the inner membrane into the mitochondrial matrix through a molecular complex and enzyme, known as ATP synthase. The flow of hydrogen ions through ATP synthase causes a conformational change in the protein, which provides the energy for the combination of ADP with inorganic phosphate to form ATP. The deposition of hydrogen ions into the intermembrane space is facilitated by energy released by the movement of electrons from Complex I (CI) to IV (CIV) of the electron transport chain (ETC) which, in turn, is dependent on the availability of O_2 at CIV to accept these electrons. Without O_2 to accept these electrons, oxidative phosphorylation cannot occur (Hatefi, 1985).

Independent of changes in mitochondrial density, qualitative alterations in the intrinsic function of the mitochondria can also impact mitochondrial ATP resynthesizing capacity and efficiency. For example, as electrons entering the ETC at CII are at a lower energy state than those entering at CI, O_2-consumption driven by CII results in the resynthesis of fewer ATP than O_2-consumption driven by CI (Lee *et al.*, 1996).

Consequently, a greater amount of O_2 is required for CII-driven respiration to resynthesize the same amount of ATP as CI-driven respiration. Intrinsic mitochondrial function may also be affected by uncoupling the relationship between O_2 consumption and ATP resynthesis. Contrary to the simplified mitochondrion illustrated in Figure 1.1, not all hydrogen ions are funneled down the gradient through ATP synthase. Some of the hydrogen ions may bypass ATP synthase by leaking through the membrane and others may achieve this by traveling through other channels, such as uncoupling proteins or the ADP/ANT translocase (Gnaiger, 2009; Larsen *et al.*, 2011; Pesta & Gnaiger, 2012). Mitochondrial O_2 consumption not coupled to ATP synthesis during *in vitro* experiments is termed State 2 respiration (Pesta & Gnaiger, 2012). Similar to altering the contributions of CI and CII, altering coupling or State 2 respiration can impact the function of the mitochondria, with greater uncoupling leading to a greater O_2 cost of ATP resynthesis (Hinkle, 2005).

Given the role mitochondria play in fueling muscular work during endurance exercise, it is not surprising that alterations in mitochondrial respiratory function have been suggested to play a role in the exercise intolerance exhibited by several patient populations, including patients with COPD (Picard *et al.*, 2008a; Naimi *et al.*, 2011; Meyer *et al.*, 2013). In addition to exhibiting severely impaired pulmonary function, patients with COPD also exhibit debilitating exercise intolerance, which is thought to be related to an increased O_2-cost of physical activity (Richardson *et al.*, 2004; Man *et al.*, 2009; Medeiros *et al.*, 2014). Indeed, diminished mitochondrial respiratory capacity related to reduced mitochondrial density likely plays a role in the exercise intolerance exhibited by patients with COPD (Picard *et al.*, 2008a; Bronstad *et al.*, 2012). However, a

mere reduction in the amount of mitochondria present in the muscle cannot account for the increased O_2-cost of exercise that accompanies the disease, suggesting that qualitative alterations in the mitochondria may also play a role in COPD. **Therefore, the second study of this dissertation investigated the possibility that qualitative alterations in mitochondrial respiration contribute to the exercise intolerance exhibited by patients with COPD.**

Mitochondrial O_2 Supply and Demand during Exercise

During incremental, dynamic exercise the rate of O_2 consumption (VO_2) increases proportionally to workload until reaching a point, known as VO_{2max}, where VO_2 fails to increase despite further increases in workload. While VO_2 can be described as the product of O_2 supply (QO_2) to the mitochondria and O_2 demand by the mitochondria (VO_2=QO_2 x Extraction), it is unclear whether O_2 supply and/or O_2 demand limit VO_{2max}. Some have hypothesized that the capacity of the cardiopulmonary system to supply O_2 to the mitochondria and the capacity of the mitochondria to consume O_2 are tightly matched, such that the capacity of neither is in excess at VO_{2max} (Hoppeler & Weibel, 1998). Alternatively, some have hypothesized that O_2 supply strongly limits VO_{2max}, such that the mitochondria are in excess at VO_{2max} (Levine, 2008; Boushel *et al.*, 2011; Boushel & Saltin, 2013). Still others have hypothesized that mitochondrial O_2 demand limits or determines VO_{2max} (Cardus *et al.*, 1998). Therefore the role of O_2 supply and O_2 demand in determining VO_{2max} is currently unclear.

O_2 Supply and O_2 Demand as determinants of VO_{2max}

Based on the concept that physiological systems are designed to match function, it has been postulated that all of the parts of the O_2 cascade are expressed in strict proportion to the task required of them at VO_{2max}, such that no component (*e.g.*, O_2 supply or O_2 demand) is in excess or deficient compared to others at VO_{2max} (Taylor & Weibel, 1981; Weibel *et al.*, 1991; Hoppeler & Weibel, 1998). This hypothesis, often referred to as the theory of symmorphosis, is supported by the tight, linear relationship between mitochondrial volume and VO_{2max} across many mammalian species (Weibel *et al.*, 1991; Hoppeler & Weibel, 1998). However, while a given increase in mitochondrial volume is typically associated with a predictable increase in VO_{2max} for most mammalian species, the VO_{2max} achieved by humans is often less than predicted based upon mitochondrial volume (Hoppeler & Weibel, 1998), which challenges the concept of symmorphosis in humans. Indeed, the application of this theory to humans is further called into question by the disproportionally small increase in VO_{2max} compared to the relatively large increase in mitochondrial capacity that typically accompanies endurance training (Gollnick *et al.*, 1973; Jacobs & Lundby, 2013). Therefore, while this theory of economic design appears to apply well to many species (Hoppeler & Weibel, 1998), it does not appear to completely describe the O_2 cascade in humans.

O_2 Supply to the Mitochondria and VO_{2max}

Some have concluded that O_2 supply limits mitochondrial O_2 consumption and subsequently VO_{2max} (Levine, 2008; Boushel *et al.*, 2011; Boushel & Saltin, 2013). Much of the evidence supporting this position comes from experiments operating under the premise that if O_2 supply acts as a bottleneck along the O_2 cascade, increasing O_2

supply should increase VO_{2max}. Indeed, multiple studies, most using physically active or endurance-trained subjects, have reported that increasing O_2 supply by having subjects breath hyperoxic gas increases VO_{2max} during maximal exercise by approximately 10% (Margaria *et al.*, 1972; Ekblom *et al.*, 1975; Knight *et al.*, 1993). Similarly, augmenting O_2 supply to the mitochondria by increasing the concentration of hemoglobin (Hb) in the blood has also been reported to augment VO_{2max} (Joyner, 2003). Based upon the high proportion of cardiac output that is distributed to the exercising muscle (Secher *et al.*, 1977) and upon comparisons between recreationally active and elite endurance-trained athletes (Levine, 2008), it has been suggested that the source of the O_2 limitation starts centrally with a limited cardiac output (Levine, 2008). Thus, it appears that O_2 supply limits mitochondrial respiratory capacity to some extent in some populations, including endurance-trained athletes.

Two general approaches have been used to quantify the extent to which O_2 supply suppresses mitochondrial respiratory capacity at VO_{2max}. The first method compares mass-specific VO_{2max} during large-muscle mass exercise to small-muscle mass exercise, during which central factors such as cardiac output are not likely to be limiting (Richardson & Saltin, 1998). Indeed, the mass-specific O_2 supply and VO_{2max} reported during KE ($_{KE}VO_2max$) are nearly double that reported for cycling exercise ($_{Cycling}VO_{2max}$) (Richardson, 2003), which suggests that the mitochondria are only operating at half-capacity at VO_{2max} during large muscle exercise.

The second method utilized to determine the extent to which O_2 supply suppresses mitochondrial respiration at VO_{2max} compares the mass-specific VO_{2max} during a given exercise to the mass-specific VO_{2max} of biopsied muscle fibers measured *in vitro*

($_{Mito}VO_{2max}$) (Rasmussen *et al.*, 2001; Boushel *et al.*, 2011). Utilizing such an approach, Boushel *et al.* (2011) reported that $_{Cycling}VO_{2max}$ was about 36% lower than $_{Mito}VO_2$max, further supporting the idea that mitochondrial O_2 consumption is markedly limited by O_2 supply. If the mitochondria are so severely limited by O_2 supply, it seems unlikely that any decrement in mitochondrial function, such as those observed in patients with COPD (Picard *et al.*, 2008a), would have any effect on exercise capacity as there would be a substantial amount of reserve capacity to buffer any such decrement in mitochondrial function. However, in this regard, it should be noted that the amount of reserve capacity reported by such studies (Richardson, 2003; Boushel *et al.*, 2011) may be somewhat exaggerated as absolute rates of O_2 consumption are commonly normalized by the entire lower-limb muscle mass, which is unlikely to be fully recruited during cycling (Lollgen *et al.*, 1980; Green & Patla, 1992).

O_2 Demand by the Mitochondria and VO_{2max}

While many studies support the concept of an O_2-supply limitation at VO_{2max}, not all studies support such a position. Interestingly, studies that provide evidence of an O_2-demand limitation appear to be most common among untrained or sedentary subjects. For example, breathing hyperoxic gas, which augmented the VO_{2max} of trained subjects (Ekblom *et al.*, 1975; Knight *et al.*, 1993), had no effect on the VO_{2max} during cycling in sedentary adults (Cardus *et al.*, 1998). Additionally, following 2 weeks of exercise training, the training-induced gain in VO_{2max} of previously sedentary subjects was related to increased mitochondrial O_2 demand and not delivery (Jacobs *et al.*, 2013). Indeed, Roca *et al.* (1992) reported that prior to endurance training, sedentary subjects were insensitive to hypoxia, whereas after training these same subjects exhibited a reduction in

exercise capacity in hypoxia. Thus, it is seems possible that physical activity, which has been reported to have disproportionate effects on mitochondrial capacity and VO_{2max} (Gollnick *et al.*, 1973), may result in a shift in the limitation to VO_{2max}, away from O_2 demand toward O_2 supply. **Consequently, the purpose of the third study of this dissertation was to determine if O_2 supply and O_2 demand similarly limit VO_{2max} in untrained and endurance-trained subjects by comparing these variables, *in vitro*, during maximal exercise with maximal *in vitro* mitochondrial O_2 consumption.**

Summary of Specific Aims of Dissertation

As the O_2 cascade plays a critical role in determining health and physical function, alterations in how this cascade supplies or demands O_2 may have significant consequences in terms of health and quality of life. Advancing a greater understanding of the regulation and factors associated with O_2 supply and demand, and how disease may affect these processes, is necessary for developing therapies and treatments for those with dysfunctional O_2 transport and utilization. Therefore, this dissertation will address the following specific aims:

1. Determine the mechanisms involved in sensing and mediating the sympatholytic effect of heat in isolated human skeletal muscle feed arteries
2. Investigate the possibility that qualitative alterations in skeletal muscle mitochondrial O_2 consumption contribute to exercise intolerance exhibited by patients with COPD
3. Determine whether O_2 supply or O_2 demand similarly limit VO_{2max} in untrained and endurance-trained subjects

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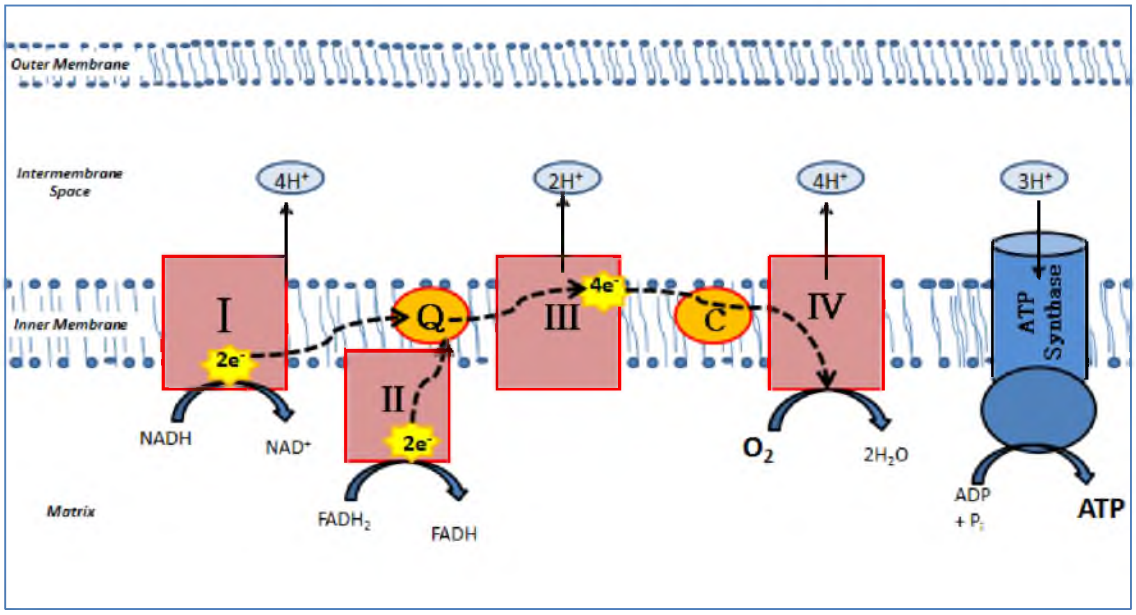

Figure 1.1: Mitochondrial electron transport chain and oxidative phosphorylation Electrons are delivered to Complex I and Complex II in the form of NADH and $FADH_2$, which are then passed on to coenzyme Q (Q), which transports the electrons on to Complex III. From Complex III, the electrons are shuttled to Complex IV by cytochrome c (C). It is at Complex IV where oxygen (O_2) is reduced (*i.e.*, consumed) by the electrons to form water. Note that as the electrons are passed from complex to complex energy is released. At Complexes I, III, and IV this energy is used to shuttle hydrogen ions (H^+) across the inner membrane into the intermembrane space. The hydrogen ions are then funneled back across the inner membrane through ATP synthase, providing the energy for the resynthesis of ATP from adenosine diphosphate (ADP) and inorganic phosphate (P_i). See Hatefi, 1985 for a more detailed description of the electron transport chain and oxidative phosphorylation.

CHAPTER 2

α_1 AND α_2 ADRENERGIC RESPONSIVENESS IN HUMAN SKELETAL MUSCLE FEED ARTERIES:THE ROLE OF TRPV ION CHANNELS IN HEAT-INDUCED SYMPATHOLYSIS

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Gifford JR, Ives SJ, Park SY, Andtbacka RHI, Hyngstrom JR,
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α_1- and α_2-Adrenergic responsiveness in human skeletal muscle feed arteries: the role of TRPV ion channels in heat-induced sympatholysis

Jayson R. Gifford,[1,2] Stephen J. Ives,[1,3] Song-Young Park,[1,2] Robert H. I. Andtbacka,[4] John R. Hyngstrom,[4] Michelle T. Mueller,[5,6] Gerald S. Treiman,[5,6] Christopher Ward,[5,6] Joel D. Trinity,[1,6] and Russell S. Richardson[1,2,6]

[1]*Geriatric Research, Education, and Clinical Center, Salt Lake City Veterans Affairs Medical Center, Salt Lake City, Utah;* [2]*Department of Exercise and Sport Science, University of Utah, Salt Lake City, Utah;* [3]*Department of Health and Exercise Sciences, Skidmore College, Saratoga Springs, New York;* [4]*Department of Surgery, Huntsman Cancer Hospital, University of Utah, Salt Lake City, Utah;* [5]*Division of Surgery, Salt Lake City Veterans Affairs Medical Center, Salt Lake City, Utah; and* [6]*Department of Internal Medicine, University of Utah, Salt Lake City, Utah*

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Gifford JR, Ives SJ, Park SY, Andtbacka RH, Hyngstrom JR, Mueller MT, Treiman GS, Ward C, Trinity JD, Richardson RS. α_1- and α_2-Adrenergic responsiveness in human skeletal muscle feed arteries: the role of TRPV ion channels in heat-induced sympatholysis. *Am J Physiol Heart Circ Physiol* 307: H1288–H1297, 2014. First published August 29, 2014; doi:10.1152/ajpheart.00068.2014.—The purpose of this study was to determine if heat inhibits α_2-adrenergic vasocontraction, similarly to α_1-adrenergic contraction, in isolated human skeletal muscle feed arteries (SMFA) and elucidate the role of the temperature-sensitive vanilloid-type transient receptor potential (TRPV) ion channels in this response. Isolated SMFA from 37 subjects were studied using wire myography. α_1 [Phenylephrine (PE)]- and α_2 [dexmedetomidine (DEX)]-contractions were induced at 37 and 39°C with and without TRPV family and TRPV4-specific inhibition [ruthenium red (RR) and RN-1734, respectively]. Endothelial function [acetylcholine (ACh)] and smooth muscle function [sodium nitroprusside (SNP) and potassium chloride (KCl)] were also assessed under these conditions. Heat and TRPV inhibition was further examined in endothelium-denuded arteries. Contraction data are reported as a percentage of maximal contraction elicited by 100 mM KCl (LT_{max}). DEX elicited a small and variable contractile response, one-fifth the magnitude of PE, which was not as clearly attenuated when heated from 37 to 39°C (12 ± 4 to 6 ± 2% LT_{max}; $P = 0.18$) as were PE-induced contractions (59 ± 5 to 24 ± 4% LT_{max}; $P < 0.05$). Both forms of TRPV inhibition restored PE-induced contraction at 39°C ($P < 0.05$) implicating these channels, particularly the TRPV4 channels, in the heat-induced attenuation of α_1-adrenergic vasocontraction. TRPV inhibition significantly blunted ACh relaxation while denudation prevented heat-induced sympatholysis without having an additive effect when combined with TRPV inhibition. In conclusion, physiological increases in temperature elicit a sympatholysis-like inhibition of α_1-adrenergic vasocontraction in human SMFA that appears to be mediated by endothelial TRPV4 ion channels.

heat; feed arteries; alpha adrenergic; sympatholysis; TRPV ion channels

DURING MUSCLE CONTRACTION, the normal vasoconstriction response to stimulation of α_1- and α_2-adrenoceptors is attenuated in a process termed functional sympatholysis (4, 25). Inspired by the theory that exercise-induced heat generation plays a role in mediating this local inhibition of α-adrenergic vasoconstriction (4), our group recently tested the effect of heat on α_1-adrenergic responsiveness in isolated human skeletal muscle feed arteries (SMFA) (14, 15). In 2011, Ives et al. (15) reported that simply heating these isolated feed arteries from 37 to 39°C (approximate temperature of muscle at rest and exercise, respectively; Ref. 28) attenuated α_1-induced adrenergic vasoconstriction by ~40%. It was later determined that this sympatholytic effect of heat could be prevented by inhibiting endothelial nitric oxide synthase (eNOS) (14), implicating nitric oxide (NO) as a key player in heat-induced sympatholysis. While these studies support the concept of heat-induced sympatholysis, they do not explain how these denervated arteries sense the increase in temperature to initiate the relaxing response.

Within the endothelial and smooth muscle cells of the vasculature exists a family of temperature-sensitive ion channels, known as the vanilloid-type transient receptor potential (TRPV) ion channels, which seems poised to serve as the link between heat exposure and sympatholysis. In fact, while investigating the function of type IV TRPV (TRPV4) ion channels Watanabe et al. (36) reported that heating cultured cells, including mouse aorta endothelial cells, to temperatures similar to those employed by Ives et al. (14, 15) resulted in an influx of calcium that was prevented by treating cells with the TRPV antagonist ruthenium red (RR) or by the genetic inhibition of the TRPV4 channels specifically. Considerable additional evidence indicates that the activation of the TRPV ion channels by other stimuli, like shear stress and endogenous ligands, can modulate vascular responsiveness and enhance endothelial-dependent dilation (2, 7, 8, 19, 21, 24, 35, 39). Therefore, given their known impact on vascular function and sensitivity to physiological temperatures (2, 6, 33), it seems plausible that the TRPV ion channels, especially TRPV4 ion channels, play a role in sensing and mediating the sympatholytic effect of heat in human SMFA (14, 15). However, this potential mechanism has yet to be elucidated.

Therefore, the purpose of this study was to better characterize α-adrenergic function in human SMFA and explore the possibility that the TRPV ion channels, particularly the TRPV4 channel, act as the link between elevated temperature and blunted α-adrenergic vasocontraction (14, 15). As the function of the α_2-adrenoceptors, which has yet to be documented in these arteries, is believed to differ from their α_1-counterparts in distribution and sensitivity to sympatholysis (18, 23), we first sought to compare the effects of α_1- and α_2-agonists on human SMFA and determine whether α_2-induced vasocontraction is

Address for reprint requests and other correspondence: R. S. Richardson, VA Medical Center Bldg 2, 500 Foothill Dr., Salt Lake City, UT 84148 (e-mail: r.richardson@hsc.utah.edu).

also inhibited in the heat. We then tested two hypotheses regarding the role of TRPV ion channels in the restoration of vascular function mediated by α_1- and α_2-adrenoceptors in the heat. Specifically, based on the observation of Watanabe et al. (36) that TRPV inhibition prevented the response of cultured endothelial cells to heat, we first hypothesized that the inhibition of these ion channels and/or endothelial denudation would result in the restoration of α-adrenergic vasocontraction at 39°C to levels observed at 37°C. Second, we tested the hypothesis that this restorative effect of TRPV inhibition would be associated with altered endothelial function and not altered smooth muscle function.

METHODS

Subjects and General Procedures

Thirty-seven diverse subjects (22 male, 15 female, 22–71 yr) undergoing routine melanoma-diagnosis-related surgery donated SMFA that were removed during the normal course of the surgery. Vessels from subjects who had undergone chemotherapy were not included in this study as this was a contraindication for the surgery. Other medical conditions and medications used by the subjects were noted, but no exclusion criteria were based on these data (Table 1). All protocols used in this study were approved by the Institutional Review Board of the University of Utah and Salt Lake City Veterans Affairs Medical Center, and informed written consent was obtained from all subjects before surgery. All protocols were carried out in accordance with the Declaration of Helsinki.

Vessel Harvest and Preparation

SMFAs from the axillary and inguinal regions were obtained during melanoma-related node dissection surgeries at the Huntsman Cancer Hospital and the Salt Lake City Veterans Affairs Hospital.

Table 1. *Subject characteristics*

	Means ± SE	Normal Range
Vital characteristics		
Age	53 ± 2	—
Male/female (*n*)	22/15	—
Height, cm	175 ± 2	—
Weight, kg	88 ± 3	—
Body mass index, kg/m^2	28 ± 1	—
Systolic blood pressure, mmHg	128 ± 3	—
Diastolic blood pressure, mmHg	78 ± 2	—
Mean arterial blood pressure, mmHg	94 ± 2	—
Blood and plasma		
Glucose, mg/dl	96 ± 3	65–110
Blood urea nitrogen, mg/dl	16 ± 1	6–21
Creatinine, mg/dl	1.0 ± 0.1	0.52–0.99
Lactate dehydrogenase, U/l	317 ± 28	313–618
Hemoglobin, g/dl	14 ± 0.3	12–16
White blood cells, ×10^3/μl	7 ± 0.3	3.6–10.6
Red blood cells, ×10^6/μl	4.7 ± 0.1	4.0–5.2
Platelets, ×10^3/μl	244 ± 12	150–400
Hematocrit, %	43 ± 1	36–46
Cardiovascular medication (users/*n*)		
Diuretic	3/37	—
Ca^{2+} channel blocker	3/37	—
β-Blocker	2/37	—
Angiotensin blocker	1/37	—
Angiotensin-converting enzyme inhibitor	5/37	—
Statin	10/37	—
Vessel location (*n*/total)		
Axillary	27/37	—
Inguinal	10/37	—

Patients were anaesthetized using a standard protocol including propofol, fentanyl, benzodiazepines, and succinylcholine. After lymph node dissection, feed arteries entering muscles in the axillary (e.g., serratus anterior, or latissimus dorsi) or inguinal (e.g., quadriceps femoris or hip adductors) regions were identified and classified as SMFA based on entry into a muscle bed, structure, blood color, and pulsatile bleed pattern. The vessels were ligated, excised, and then immediately placed in cold (~4°C), normal physiological saline solution (NPSS), which consisted of 125 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 18 mM $NaHCO_3$, 0.026 mM Na_2EDTA, and 11.2 mM glucose. Note that the pH of the NPSS in the bath was maintained at 7.4 ± 0.5 at all temperatures throughout the entirety of the experiment. Vessels were then brought to the laboratory for experimentation within 15 min of harvesting.

Once at the laboratory, feed arteries were dissected (to remove all perivascular adipose and other tissue) under a stereomicroscope and cut into six to eight ring segments (~2 mm in length) while immersed in cold NPSS. Rings were then mounted in wire myography chambers (700 MO; DMT Systems, Aarhus, Denmark) in cold NPSS that was continuously aerated with carbogen gas (95% O_2–5% CO_2) throughout the experiment. The diameter and length of the mounted rings were assessed using a calibrated micrometer eyepiece. Myography chambers were then gradually warmed to a baseline temperature of 37°C during a 30-min equilibration period before beginning the protocol. During the protocol, NPSS was exchanged every 15 min except during the concentration-response curves described below.

Vessel Function Protocols

Before the administration of TRPV inhibitors all artery segments underwent a length-tension protocol (15) at 37°C to assure that the arteries were at the length (L_{max}) that would elicit the greatest contractile response to a single exposure to 100 mM KCl dissolved in NPSS (LT_{max}). Specifically, LT_{max} was reached when increasing the length of the arteries increased the response to 100 mM KCl by <10% (15). Following the length tension protocol, half of the rings were continuously incubated with a TRPV antagonist to assess the role of the TRPV ion channels in modulating the responsiveness of the arteries. Given that multiple TRPV ion channels may potentially be involved in mediating the sympatholytic effect of heat, RR (30 μM) (29), a nonspecific TRPV antagonist (6, 29), was applied to the arteries to inhibit the activity of the family of TRPV channels. As the TRPV4 ion channel has been reported to be activated by similar levels of heat, as utilized in this study, previously in arterial preparations (36), the selective TRPV4 antagonist RN-1734 (20 μM) (3, 34) was applied to a different set of arteries (n = 7) to explore the role of this specific ion channel in the sympatholytic effect of heat.

After allowing the treated rings to incubate in RR or RN-1734 for 15–20 min, α-adrenergic responsiveness of all rings (TRPV inhibited and control rings) was assessed at 37°C by generating concentration-response curves to the α_1-agonist phenylephrine (PE; 10^{-9}-10^{-3} logM) and the α_2-agonist dexmedetomidine (DEX; 10^{-10}-10^{-3} logM). Due to the variability of individual vessel ring caliber, vasocontractile responses for each ring were normalized to the maximum response to 100 mM KCl during the length tension protocol (i.e., LT_{max}) (15). As described previously (14), the endothelium-dependent vasorelaxation was assessed with concentration-response curves to acetylcholine (ACh; 10^{-7}-10^{-3} logM) following preconstriction with PE to ~70% of the maximum PE response. Note that the order of the PE and DEX concentration-response curves was alternated in a balanced manner to account for any ordering effect.

The chambers were then heated from 37 to 39°C over a period of 10 min. After a 20- to 30-min equilibration period, the concentration-response curves for PE, DEX, and ACh were performed once again as described above. As prior exposure to heat has been reported to alter adrenergic responsiveness (27) we, like others (15, 20), always performed the highest temperature phase last. Given the potential for a

time effect in this design, we performed a time control experiment in a set of six arteries by generating concentration-response curves to PE (10^{-9}-10^{-3} logM), with the temperature held constant at 37°C at *hour 1* and *hour 2* of the experiment in the presence and absence of RR.

As previous research has suggested that the sympatholytic effect of heat may be mediated by the endothelium (14), we also sought to determine whether the effect of the TRPV ion channels on vascular function was endothelium dependent by performing the control and RR experiments in endothelium denuded arteries ($n = 6$). Arteries were denuded by passing 2 ml of air through the lumen of the artery before it was dissected and mounted on the wire myograph (22). Once mounted, denudation was verified by the vessel rings exhibiting <10% relaxation to ACh (10^{-3}) at 37°C.

Another set of arteries was used to assess the effect of heat and TRPV inhibition on smooth muscle function. Following the length tension protocol described earlier, rings were rinsed with NPSS and allowed to recover to baseline tension. To test receptor-independent smooth muscle vasocontraction with and without TRPV inhibition, KCl was added to the NPSS buffer at 37 and 39°C to progressively raise the concentration of KCl in the bath from 10 to 100 μM, with and without RR. Endothelium-independent relaxation was assessed with concentration-response curves to the NO donor sodium nitroprusside (SNP; 10^{-9}-10^{-4} logM) in all conditions (37 and 39°C with and without RR) following preconstriction with PE. As pilot studies suggested that prior exposure to SNP may alter vascular responsiveness, each ring used in the SNP protocol was exposed to only one of the four conditions (37 or 39°C, with or without RR).

Statistical Analyses

All myography tension data were acquired at 4 Hz with an analog-to-digital data acquisition system (Biopac Systems, Goleta, CA). As described above, unless otherwise stated, contraction data are represented as a percentage of the maximum contraction elicited by 100 mM KCl during the length tension protocol (i.e., $\%LT_{max}$) for each arterial ring, and relaxation data are represented as the magnitude of relaxation from preconstriction tension divided by the magnitude of preconstriction tension for each arterial ring (i.e., %relaxation). As the arterial rings do not always reach peak relaxation or tension at the highest drug concentration, maximum relaxation and contraction data, regardless of concentration, are presented in addition to the concentration-response curve data. Sensitivity of the arteries to PE, DEX, KCl, and SNP was assessed by calculating the $logEC_{50}$ with a sigmoidal parameter $\{(a + b - a)/[1 + 10^{(x-c)}]\}$ as described previously (15).

Statistical analyses were performed using SPSS statistical software (SPSS version 17; SPSS, Chicago, IL), while graphs and figures were created using Sigma Plot version 11 (Systat Software, San Jose, CA). Note that values presented in text and figures are means ± SE, unless otherwise stated. Repeated-measures ANOVA followed by Tukey's post hoc test was used to determine if α_1- and α_2-stimulation resulted in significant contractions above baseline levels. A paired *t*-test was used to examine differences between maximal α_1- and α_2-induced vasocontraction. Repeated-measures ANOVA followed by Tukey's post hoc test was used to examine the effect of temperature and TRPV inhibition and denudation on arterial responsiveness for each concentration-response curve, maximum response, and $logEC_{50}$. The level of significance for all tests was set a priori at $\alpha = 0.05$.

RESULTS

Vessel Characteristics

Twenty-seven SMFA from the axillary region and ten SMFA from the inguinal region were harvested for this study. The average internal diameter of the feed arteries was 515 ± 40 μm. The maximum response to the length tension protocol (LT_{max}) was 1,076 ± 135 mg at L_{max}, with a starting tension of 1,254 ± 82 mg.

Vessel Function

α_1- and α_2-Adrenergic responsiveness. At 37°C, exposure to the α_1-agonist PE and the α_2 agonist DEX resulted in significant contractions above baseline tension ($P < 0.05$), with higher concentrations of DEX tending to elicit relaxation from the tension generated in response to lower concentrations of DEX (Fig. 1). Interestingly, responsiveness to DEX proved to be more varied from artery to artery with many arteries exhibiting little-to-no response to DEX while several others exhibited strong responses to DEX (~45% LT_{max}). This variance was not accounted for by age nor gender, although females did tend to have a greater response to DEX than males (17 ± 6 and 8 ± 3, respectively, $P = 0.18$). Nevertheless, Fig. 1 illustrates that on average the magnitude of maximal α_2-induced contractions was far less than the magnitude of the maximal α_1-induced contractions (59 ± 5 and 12 ± 4% LT_{max}, respectively; $P < 0.001$).

Effect of TRPV inhibition and heat on α-adrenergic function. Treating arteries with RR or RN-1734 had no significant effect on baseline tension at 37°C ($P > 0.05$). Heating human SMFA from 37 to 39°C resulted in a small, yet significant, increase in baseline tension (35 ± 15 mg; $P < 0.05$). While statistically significant, it should be noted that this change in basal tension only represents an increase equivalent to 3% LT_{max}. Interestingly, inhibition of the TRPV ion channels prevented the heat-related increase in baseline tension such that no significant change in basal tone was observed when heated in the presence of RR (−8 ± 6 mg; $P > 0.05$).

As illustrated in Fig. 1, α_1-adrenergic responsiveness, as assessed by concentration-response curves with PE, was significantly attenuated at 39°C (24 ± 4% LT_{max}) compared with the 37°C control condition ($P < 0.05$). α_2-Adrenergic contraction with DEX also exhibited a similar tendency for decreased vasocontraction when comparing 37 to 39°C (12 ± 4, 6 ± 2% LT_{max}), but due to the already recognized variability in the responses, this heat-induced sympatholysis did not reach statistical significance ($P = 0.18$). As hypothesized, treating arteries with RR restored α_1-adrenergic contraction at 39°C to levels observed under the 37°C control condition, such that there was no significant difference between the 37°C control response curve and the 39°C RR curve ($P > 0.05$) or maximum contraction elicited by the drugs (PE: 48 ± 7% LT_{max}, and DEX: 14 ± 3% LT_{max} respectively; $P > 0.05$). Similarly, no evidence of heat-induced sympatholysis of α-adrenergic vasocontraction at 39°C was observed in arteries treated with RN-1734 (65 ± 11% LT_{max}; Fig. 1, *C* and *D*).

To determine if the restorative effect of RR and RN-1734 on the heat-induced attenuation of α-adrenergic vasocontraction was due to the blocking of the sympatholytic effect of heat or if it was acting through some other mechanism, we also examined the effect of the drugs on adrenergic responsiveness of the arterial rings at 37°C (Fig. 1). Initially, due to a tendency that failed to reach statistical significance, it appeared that PE- and DEX-induced vasocontraction may be potentiated by TRPV inhibition at 37°C, but, despite raising the *n* to increase statistical power, no significant effect of RR on PE- or DEX-induced vasocontraction at 37°C was observed ($P > 0.30$). It

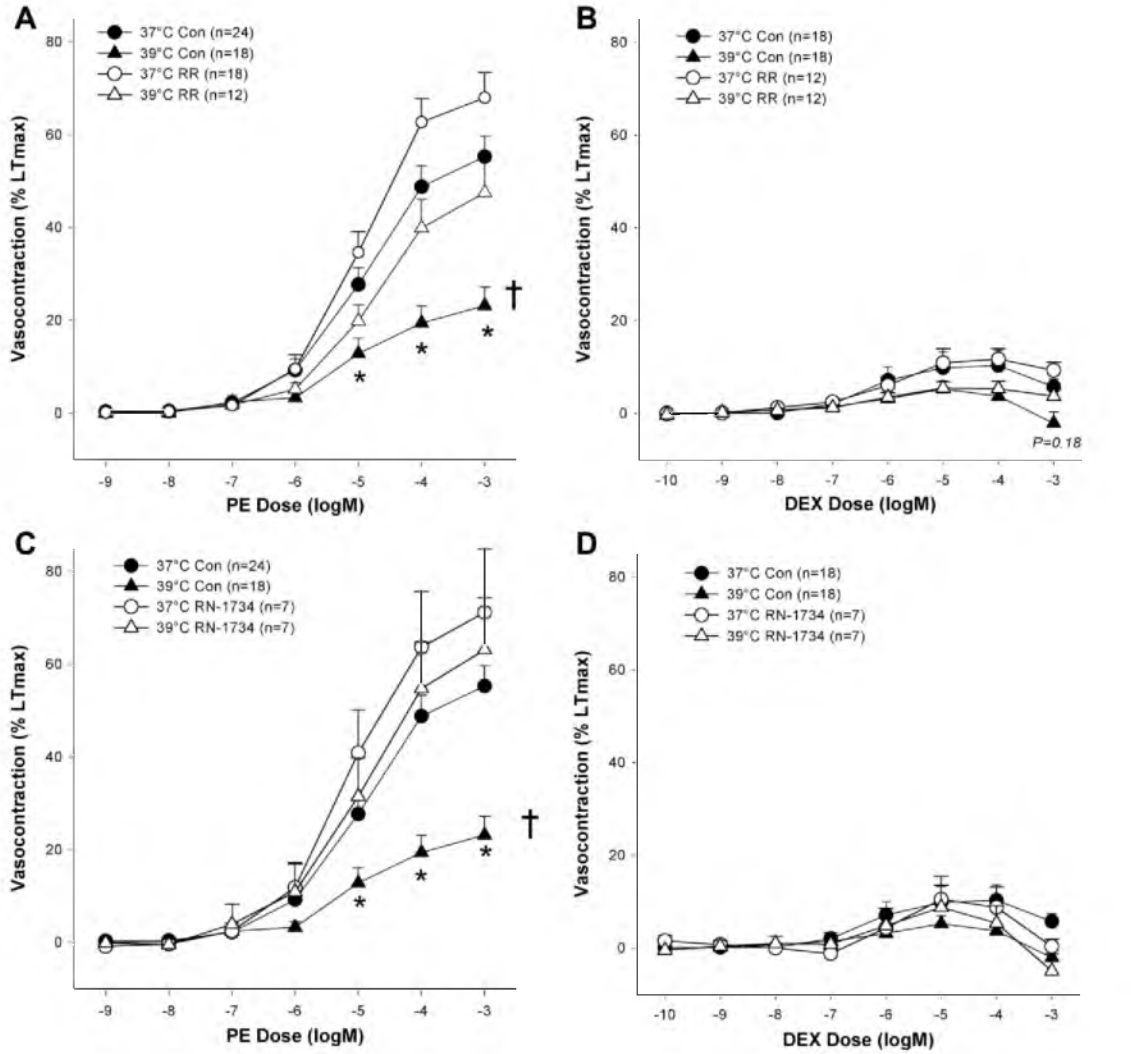

Fig. 1. Effect of temperature with and without vanilloid-type transient receptor potential (TRPV) family and TRPV4-specific inhibition [ruthenium red (RR) and RN-1734, respectively] on α_1 [phenylephrine (PE)]- and α_2 [dexmedetomidine (DEX)]-induced adrenergic vasocontraction. *A*: effect of temperature and RR on PE concentration-response curve. *B*: effect of temperature and RR on DEX concentration-response curve. *C*: effect of temperature and RN-1734 on PE concentration-response curve. *D*: effect of temperature and RN-1734 on DEX concentration-response curve. *Response to concentration significantly different than same concentration in the 37°C control condition ($P < 0.05$). †Concentration-response curve significantly different than 37°C control curve ($P < 0.05$).

should also be noted that neither temperature nor treatment with RR or RN-1734 elicited significant differences between any other pairings besides those including the 39°C control condition and did not significantly alter the sensitivity of the arteries (i.e., $\log EC_{50}$), which averaged -4.9 ± 0.1 logM for PE and -7.3 ± 0.2 logM for DEX.

Effect of heat and TRPV inhibition on endothelial function. As illustrated in Fig. 2*A*, heating from 37 to 39°C tended to augment but ultimately had no significant effect on endothelial-dependent relaxation induced by exposure to ACh (maximum relaxation: 65 ± 5 and $75 \pm 8\%$, respectively; $P > 0.05$) while the application of **RR** significantly and strongly attenuated ACh-induced vasorelaxation compared with control condition at both 37 and 39°C ($P < 0.05$). It is worth noting that relaxation in the presence of RR was similarly blunted at 37 and 39°C such that no significant difference between the two conditions was observed (maximal relaxation: 38 ± 4 and $32 \pm 5\%$; $P > 0.05$). As illustrated in Fig. 2*B*, inhibition of the TRPV4 channels with RN-1734 yielded a similar significant attenuation of ACh-induced dilation at 37°C ($20 \pm 8\%$ relaxation) and 39°C ($17 \pm 7\%$ relaxation). As TRPV inhibition appeared to affect endothelial function, ACh-induced dilation with RR was tested in endothelial-denuded arteries (Fig. 2, *C* and *D*). There was no significant difference in ACh-induced

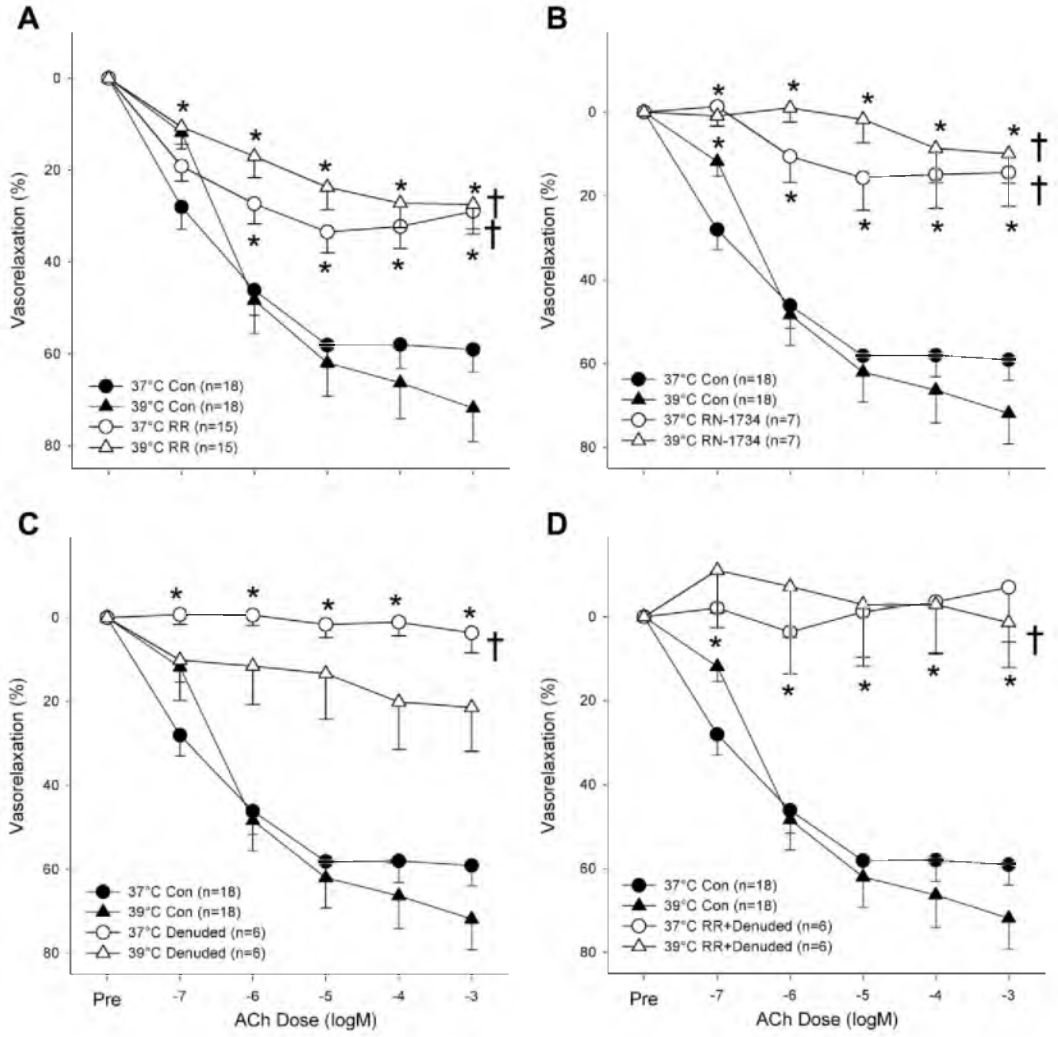

Fig. 2. Effect of heating with and without TRPV family and TRPV4-specific inhibition (RR and RN-1734, respectively) on endothelium-dependent, acetylcholine-induced (ACh) vasorelaxation. *A*: effect of heat and RR on ACh concentration-response curve. *B*: effect of heat and RN-1734 on ACh concentration-response curve. *C*: effect of heat, RR, and endothelial denudation on ACh concentration-response curve. *D*: effect of heat, RR, and endothelial denudation on ACh concentration-response curve. *Response to concentration significantly different than the same concentration in the 37°C control condition ($P < 0.05$). †Concentration-response curve significantly different than 37°C control curve ($P < 0.05$).

dilation between endothelial-denuded arteries with or without RR.

Like TRPV inhibition, endothelial denudation prevented the sympatholytic effect of heat on α_1-adrenergic contraction (Fig. 3*A*; $P > 0.05$). Furthermore, no additive effect of TRPV inhibition was revealed in endothelium-denuded arteries suggesting that the effect of the TRPV ion channels in this response is largely endothelially mediated. Endothelial denudation with or without RR had no statistically discernible effect on α_2-adrenergic contraction at 37 or 39°C (Fig. 3, *B* and *D*). Additionally, it should be noted that neither temperature nor treatment with RR or RN-1734 elicited significant differences between any other pairings besides those including the control condition.

Effect of heat and TRPV inhibition on smooth muscle function. Figure 4 illustrates that receptor-independent vasocontraction (KCl) was unaffected by heating from 37 to 39°C (maximum contraction: 94 ± 5 and 97 ± 6% LT_{max} respectively; $P > 0.05$). Additionally, the inhibition of the TRPV channels with RR did not significantly alter KCl-induced contraction at 37°C (maximum contraction: 95 ± 4% LT_{max}; $P > 0.05$) or 39°C (maximum contraction: 95 ± 6% LT_{max}; $P > 0.05$). Neither heat nor TRPV inhibition significantly altered the sensitivity of the arteries to KCl (EC_{50} = 34 ± 3

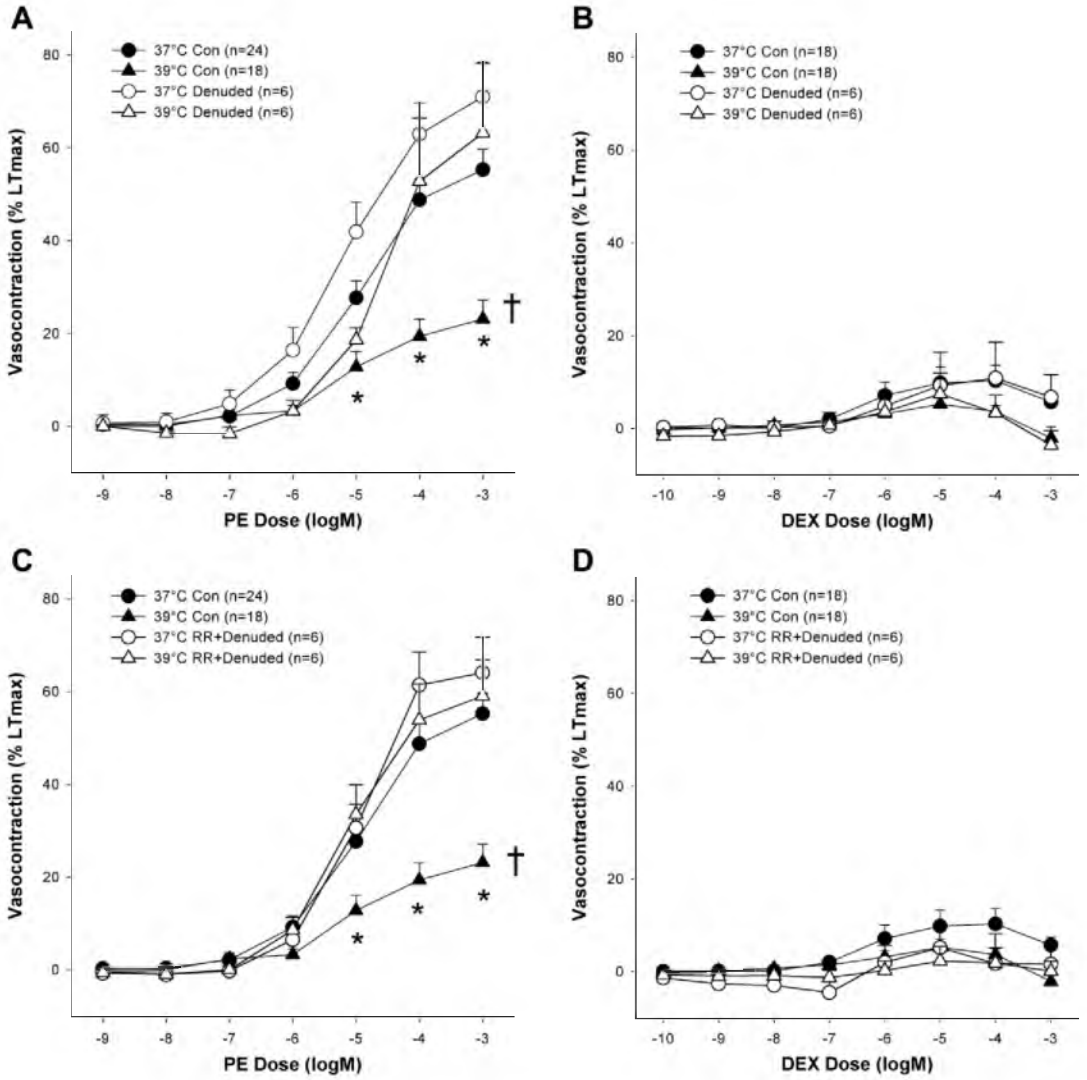

Fig. 3. Effect of endothelial denudation and TRPV family ion channel inhibition (RR) on heat-induced sympatholysis of α_1 (PE) and α_2 [dexmedetomidine (DEX)] adrenergic vasocontraction. *A*: effect of temperature and endothelial denudation on the PE concentration-response curve. *B*: effect of temperature and endothelial denudation on the DEX concentration-response curve. *C*: effect of temperature and nonspecific TRPV inhibition (RR) with endothelial denudation on the PE concentration-response curve. *D*: effect of temperature and nonspecific TRPV inhibition (RR) with endothelial denudation on the DEX concentration-response curve. *Response to concentration significantly different than same concentration in the 37°C control condition ($P < 0.05$). †Concentration-response curve significantly different than 37°C control curve ($P < 0.05$).

mM). Likewise, neither temperature nor RR significantly affected endothelium-independent relaxation as assessed by cumulative exposures to SNP ($P > 0.05$). Finally, as illustrated in Fig. 5, time had no significant effect on PE-induced vasocontraction ($P > 0.05$).

DISCUSSION

In this study we sought to better characterize α-adrenergic function in human SMFA and determine if the temperature-sensitive TRPV ion channels act as the mechanistic link between elevated temperature and attenuated α-adrenergic responsiveness in these arteries. This study resulted in three novel observations. First, we described the relative roles of the α_1- and α_2-adrenoceptors in human SMFA and documented that α_2-mediated vasocontraction is not as clearly inhibited by heat as α_1-mediated vasocontraction. Second, we observed that inhibiting the TRPV ion channels, particularly the TRPV4 channel, results in the restoration of α_1-adrenergic vasocontraction at 39°C, thereby implicating this channel in the sympatholytic effect of heat. Third, we determined that heat-

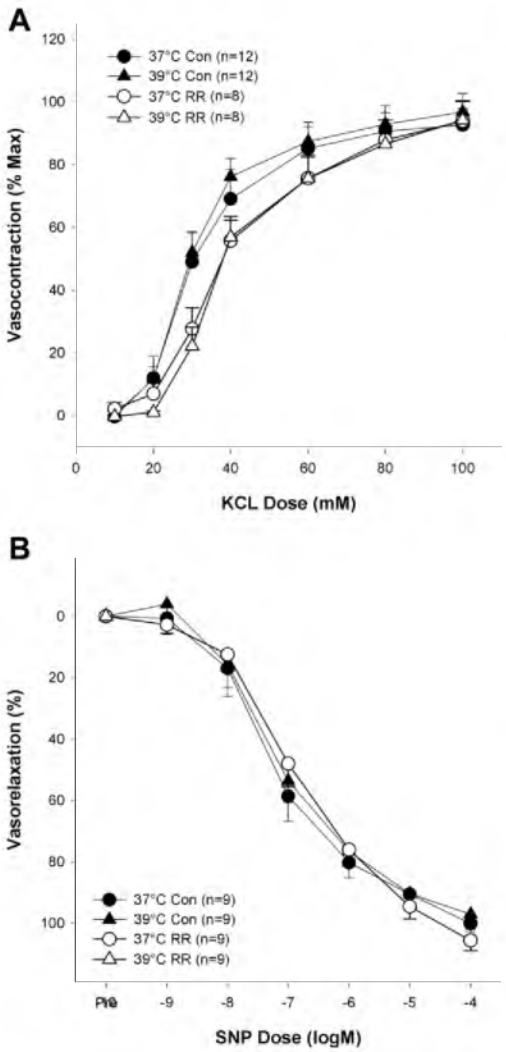

Fig. 4. Effect of heating and TRPV ion channel inhibition (RR) on smooth muscle function. *A*: effect of heat and RR on endothelium-independent vasocontraction [potassium chloride (KCl)] concentration-response curve. *B*: effect of heating and RR on endothelial independent vasorelaxation [sodium nitroprusside (SNP)] concentration-response curve.

induced sympatholysis, and the role of the TRPV ion channels in this phenomenon, occur in an endothelium-dependent manner.

Characterization α_1- and α_2-Vasocontraction in Human SMFA

Others have reported that the contribution of α_2-mediated constriction is greater in distal resistance arterioles than in proximal conduit arteries (38). With the feed artery lying somewhere between the proximal conduit and distal resistance arteries (16, 17, 30), the role of the α_2-receptors in human SMFA is unknown. Therefore, we performed concentration-response curves with both α_1- and α_2-agonists (PE and DEX, respectively) to determine what contribution the α_2-receptors make at the level of the feed artery. Indeed, the stimulation of α_2-adrenoceptors resulted in significant vasocontraction above baseline; however, the magnitude of responsiveness varied dramatically from artery to artery with some exhibiting little-to-no response to DEX and others exhibiting robust responses on the order of ~45% LT_{max}. Additionally, among many subjects higher concentrations of DEX ($>10^{-4}$ M) tended to result in relaxation and not constriction, as observed at the lower concentrations. The reason for this large variability is unclear but may possibly be related to the heterogeneity among

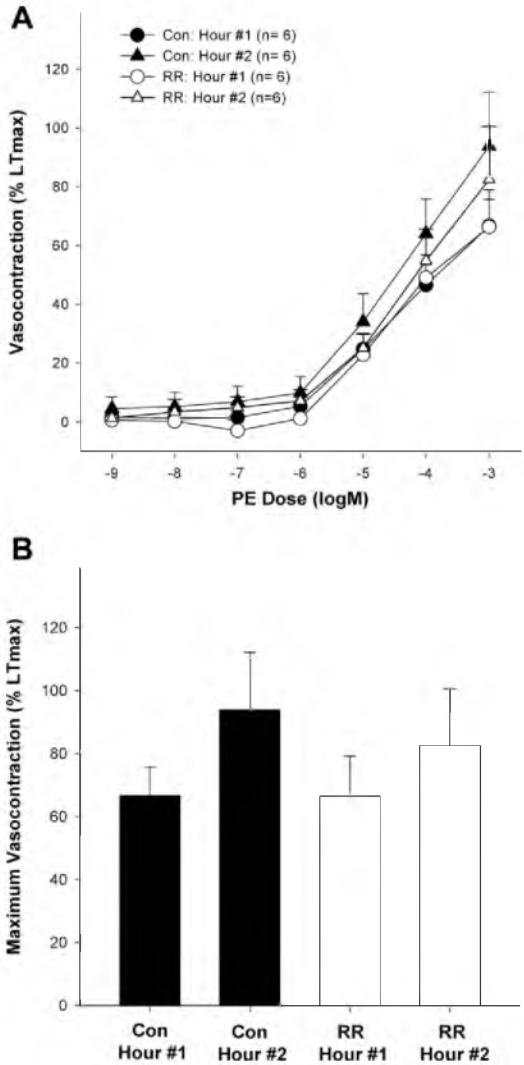

Fig. 5. Effect of time on α_1 (PE)-induced arterial responsiveness with and without TRPV family inhibition (RR). *A*: effect of time on concentration-response curve to PE with and without RR. *B*: effect of time on maximum response to PE with and without RR.

subjects participating in this study who varied considerably in terms of sex, age, and health history (31). Despite the variability of responses, on average the magnitude of the maximum α_2-induced contraction was relatively small, amounting to approximately only 20% of the magnitude of maximum α_1-induced vasocontraction (Fig. 1, *A* and *B*). It is possible that the in vitro isolation itself may have diminished the responsiveness of the α_2-adrenoreceptors as others have reported that in vitro isolation of arteries decreases α_2-adrenergic contraction by ~60% (13). However, it is important to note that even if our in vitro response was 40% of what would be observed in vivo, the corrected maximal α_2-adrenergic contraction would still amount to only 45% of its α_1-counterpart. Therefore, our data indicate that the α_2-adrenoceptor plays less of a role than the α_1-adrenoceptor in regulating the diameter and tone of human SMFA.

Much controversy exists as to the extent to which each α-receptor subtype is inhibited under sympatholytic conditions. Several studies have reported that α_1-adrenoceptors are insensitive or less sensitive to sympatholysis than their α_2-counterparts (1, 5, 32, 38) while others have reported that both α-subtypes are equally sensitive to sympatholysis (26). As illustrated in Fig. 1, both α_1- and α_2-induced vasocontraction tended to be inhibited when heated to 39°C, but only α_1-induced contraction was significantly inhibited by the heat. At first glance it seems that the α_2-receptors are not sensitive to heat-induced sympatholysis as they were not significantly inhibited at 39°C; however, upon further examination the tendency for heat-induced sympatholysis is evident in the α_2-induced contraction (Fig. 1, *B* and *D*). Thus it seems possible that the lack of a significant attenuation of α_2-induced vasocontraction may be more related to the relatively small and varied vasocontraction associated with α_2-induced contractions rather than a lack of sensitivity to heat. Further research is needed to determine if these adrenoceptors do, in fact, differ in terms of sensitivity to heat. Additionally, the varied nature of the DEX response may have also obscured the effect of other conditions like TRPV inhibition and denudation on α_2-induced vasocontraction, which revealed no clear trends.

Role of TRPV Channels in Heat-induced Sympatholysis of Adrenergic Vasocontraction

Given their known temperature sensitivity (2, 6, 11, 21, 36) and interaction with vascular function ranging from coronary to cutaneous circulation (7, 10, 21, 37, 40), we hypothesized that the TRPV ion channels would serve as the link between elevated temperature and attenuated α-adrenergic contraction in isolated human SMFA. Indeed, as illustrated in Fig. 1*A*, the inhibition of the TRPV ion channel family with RR restored α_1-adrenergic vasocontraction at 39°C to levels observed at 37°C implying that the TRPV ion channels play a role in mediating heat-induced sympatholysis. Such inhibition had no significant effect on α_2-adrenergic vasocontraction (Fig. 1*B*). Having observed that nonspecific TRPV inhibition restored α_1-adrenergic contraction in the heat, we sought to determine if a single member of the TRPV family could be implicated in this response. Given their sensitivity to temperatures ranging from 25 to 39+°C (2) and their previously described relationship with endothelium-dependent dilation (21, 41), we explored the possibility that the TRPV4 ion channel mediates the sympatholytic effect of heat. As illustrated in Fig. 1*C*, similar to arteries treated with nonspecific TRPV inhibition, arteries treated with the TRPV4-specific inhibitor RN-1734 exhibited normal levels of vasocontraction in the face of the heat stimulus. Thus these data indicate that the TRPV4 ion channel is specifically involved in mediating the sympatholytic effect of heat, which is in agreement with previous research that has documented that heating cultured rat aortic endothelial cells elicits an intracellular calcium flux that was prevented by inhibiting the TRPV4 channels (36). Thus it appears that the TRPV4 ion channel senses, either directly or indirectly (2, 36), the increase in temperature and subsequently initiates the sympatholytic effect of heat. Interestingly, although TRPV inhibition at 37°C tended to augment adrenergic contraction in some cases, this did not do occur consistently or significantly suggesting that the TRPV channels have only a minor influence on α_1- and α_2-induced vasocontraction under seemingly normothermic conditions.

Potential Mechanisms of Involvement of TRPV Channels in Heat-Induced Sympatholysis

As we have previously determined that heat-induced sympatholysis is the result of increased NO production (14), it seems likely that the activation of the TRPV4 ion channels with heat attenuates adrenergic vasocontraction by precipitating an increase in endothelial NO production. The likelihood of this potential explanation is supported by several lines of evidence. Using isolated endothelial cells and arteries from rats, Kohler et al. (21) found that applying moderate warmth (~37°C) increased the activity of the TRPV4 ion channels and reported that such an increase in activity did in fact augment NO-dependent vasodilation. Furthermore, the putative relationship between TRPV activation and NO production is supported by reports that the activation of TRPV1 ion channels increases eNOS activity and NO production in isolated skeletal muscle arterioles and endothelial cells (7, 19).

Although we did not measure NO production in the current study, we did probe the role of the endothelium in this phenomenon by assessing endothelial-dependent vasorelaxation with ACh and examining the effect of heat on endothelium-denuded arteries. As illustrated in Fig. 2, when the TRPV ion channels were inhibited, with RR or RN-1734, ACh-induced relaxation was potently attenuated thereby supporting the relationship between the TRPV channels and the endothelium that has been reported previously (21). If the TRPV channels in the endothelium initiated that sympatholytic effect of heat, it stands to reason that one would find augmented ACh-induced vasorelaxation in the heated condition. Interestingly, although such a tendency existed, we did not observe a significant potentiation of ACh-induced relaxation, possibly due to the heterogeneity among subjects. Nevertheless, more concrete evidence that the TRPV ion channels mediate the sympatholytic effect of heat in an endothelium-dependent manner comes from the current experiments that indicated that the endothelium is vital for the sympatholytic effect of heat. Specifically, that TRPV inhibition in conjunction with endothelial denudation had no additive effect on the response to PE in denuded arteries (Fig. 3). Considering that denudation prevented the sympatholytic effect of heat, and that TRPV inhibition attenuated endothelium-dependent relaxation while preventing the

sympatholytic effect of heat, it seems likely that the TRPV4 ion channels mediate the response in an endothelium-dependent manner and not by way of altered smooth muscle function, which was not significantly affected by heat and TRPV inhibition (Fig. 4).

It is worth noting that, like eNOS inhibition (14), denudation had no significant effect on α_1- or α_2-vasocontraction at 37°C indicating that endothelial activity does not significantly suppress adrenergic contraction under normothermic conditions. Additionally, it is important to note that the arteries in this preparation were studied in isolation, thus precluding a role of the central nervous system in the sympatholytic effect of heat. Nevertheless, it should be noted that even with careful dissection it is likely impossible to completely remove all nerve fibers from the SMFA and therefore there remains the possibility that TRPV ion channels on sensory and/or sympathetic nerve endings embedded in the artery wall mediate the vascular response in a manner similar to the axon reflex that mediates cutaneous hyperemia in response to local heating (12, 37).

Experimental Considerations

As prior exposure to heat has been reported to alter adrenergic responsiveness (27), we, like others (15, 20), performed the highest temperature phase last. Recognizing that with this design the observed decreases in adrenergic responsiveness may be due to time and not temperature, we performed a time control experiment with PE in SMFA. As illustrated in Fig. 5, and in agreement with prior work (15), adrenergic vasoconstriction was preserved over the duration of the experiment indicating that time was likely not a factor in the decreased adrenergic responsiveness we observed with heating. Due to the limited supply of human SMFA we limited the time control experiment to PE-induced contractions reasoning that if time had an effect on adrenergic contraction it would likely affect PE-induced and DEX-induced contraction similarly.

Based on previous research (36), we hypothesized that the TRPV4 ion channel would mediate the sympatholytic response to heat, but given the potential overlap in temperature sensitivity by other members of the TRPV family we could not rule out the possibility that other TRPV ion channels were involved in mediating the response. Therefore, we used the chemical RR, a nonspecific TRPV ion channel inhibitor, to screen for the role of the TRPV family in the sympatholytic effect of heat. Once the data using RR pointed to a role for TRPV ion channels, a more specific inhibitor, RN-1734, was utilized to pinpoint which TRPV channel was involved. With regard to RR it is important to consider that it is not completely specific to the TRPV ion channels and may affect other channels like the cold-sensitive TRPM8 channels, which are not likely to be active under the warm conditions of our experiment, and ryanodine receptors (8). Thus the possibility exists that these other channels may account, in part, for some of the results of this experiment. Nevertheless, the response to stimuli, like heat, by tissues treated with RR and by tissues in which the TRPV channels have been genetically nullified, document good agreement (9, 24, 36). Additionally, it should be noted that in the current study there was good agreement between the effects of RR and RN-1734 and there was no significant impact of RR on baseline tension, like in the work of Kohler et al. (21). Thus it appears that RR had the majority of its effects on the heat-sensitive TRPV ion channels and not on other putative targets of the drug.

As documented in Table 1, the subjects participating in this study were heterogeneous in terms of age, sex, and health history and therefore do not reflect a specific subset of the population but rather represent humans as a whole. Furthermore, although the SMFA were harvested during sentinel node biopsies, performed to determine if melanoma has spread from the skin, this was of little significance as, in the majority of these cases, the cancer was determined not to have had systemic consequences. Thus the conclusions of this study are likely applicable to the population as whole.

Conclusions

In this study we sought to describe the functional roles of α_1- and α_2-adrenoceptors in SMFA and explore the possibility that the TRPV ion channels, particularly the TRPV4 channel, act as the link between elevated temperature and blunted α-adrenergic vasocontraction. We conclude that the contribution of the α_2-receptors in human SMFA is minimal, more varied, and not as clearly inhibited by heat compared with the α_1-receptors. As hypothesized, the inhibition of TRPV ion channels, particularly the TRPV4 channels, restored α_1-adrenergic vasocontraction at 39°C to levels observed at 37°C, thereby implicating the TRPV ion channels as a component of the heat-induced sympatholysis acting in an endothelial-dependent manner. Thus it appears that physiological increases in temperature, typical of the muscle bed during moderate intensity exercise, likely activate the endothelial TRPV4 ion channels to modulate vascular function, ultimately yielding a sympatholysis-like attenuation of α-adrenergic vasocontraction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: J.R.G., S.J.I., and R.S.R. conception and design of research; J.R.G., S.J.I., S.-Y.P., R.H.A., J.R.H., M.T.M., G.S.T., and J.D.T. performed experiments; J.R.G., S.J.I., and R.S.R. analyzed data; J.R.G., S.J.I., S.-Y.P., C.W., and R.S.R. interpreted results of experiments; J.R.G. prepared figures; J.R.G. and R.S.R. drafted manuscript; J.R.G., S.J.I., S.-Y.P., R.H.A., J.R.H., M.T.M., G.S.T., C.W., J.D.T., and R.S.R. edited and revised manuscript; J.R.G., S.J.I., S.-Y.P., R.H.A., J.R.H., M.T.M., G.S.T., C.W., J.D.T., and R.S.R. approved final version of manuscript.

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CHAPTER 3

EXERCISE INTOLERANCE IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE: THE ROLE OF ALTERED SKELETAL MUSCLE MITOCHONDRIAL RESPIRATION

Abstract

This study sought to determine if qualitative alterations in skeletal muscle mitochondrial respiration, associated with decreased mitochondrial efficiency, contribute to exercise intolerance in patients with COPD. Using permeabilized muscle fibers from the vastus lateralis of 13 patients with COPD and 12 healthy controls, Complex I (CI) and Complex II (CII)-driven State 3 mitochondrial respiration were measured separately (State 3:CI and State 3:CII) and in combination (State 3:CI+CII). State 2 respiration was also measured. Exercise tolerance was assessed by knee extensor exercise (KE) time to fatigue. Per mg of muscle, State 3:CI+CII and State 3:CI were reduced in COPD ($P<0.05$), while State 3:CII and State 2 were not different between groups. To determine if this altered pattern of respiration represented qualitative changes in mitochondrial function, respiration states were examined as percentages of peak respiration (State 3:CI+CII), which revealed altered contributions from State 3:CI (Con: 83.7 ± 3.4 COPD: 72.1 ± 2.4 %Peak, $P<0.05$) and State 3:CII (Con: 64.9± 3.2 COPD: 79.5 ± 3.0 %Peak, $P<0.05$) respiration, but not State 2 respiration in COPD. Importantly, a diminished contribution of CI-driven respiration relative to the metabolically less-efficient CII-driven respiration (CI/CII) was also observed in COPD (Con: 1.28 ± 0.09, COPD: 0.81 ± 0.05, $P<0.05$), which was related to exercise tolerance of the patients ($r=0.64$, $P<0.05$). Overall, this study indicates that COPD is associated with qualitative alterations in skeletal muscle mitochondria that affect the contribution of CI and CII-driven respiration, which potentially contributes to the exercise intolerance associated with this disease.

Introduction

Exercise intolerance and an increased oxygen (O_2)-cost of physical activity are common features of COPD and are related to diminished quality of life and increased mortality in this population (Richardson *et al.*, 2004; Man *et al.*, 2009; Medeiros *et al.*, 2014). While impaired lung function certainly plays a role in exercise intolerance, the fact that diminished physical function persists even after lung function is restored by transplantation or other therapeutic means (Lands *et al.*, 1999; Amann *et al.*, 2010; Bartels *et al.*, 2011) indicates that mechanisms peripheral to the pulmonary system are involved in this dysfunction. Impaired mechanical efficiency (*i.e.*, increased ATP or O_2 cost of contraction) has been documented in the exercising muscle of patients with COPD (Baarends *et al.*, 1997; Richardson *et al.*, 2004; Medeiros *et al.*, 2014) and is thought to contribute to this debilitation. However, the underlying mechanisms involved in this decreased mechanical efficiency and their relationship to exercise intolerance in COPD remain uncertain.

During exercise, mechanical efficiency is influenced by both the efficiency of the mitochondria to resynthesize ATP (*i.e.*, mitochondrial efficiency) and the efficiency of the muscle to translate ATP into mechanical work (*i.e.*, ATP cost of contraction) (Whipp & Wasserman, 1969). Our group recently reported that patients with COPD exhibit an increased ATP cost of muscle contraction which likely contributes to diminished mechanical efficiency (Layec *et al.*, 2011; Layec *et al.*, 2012). However, mitochondrial efficiency, which can be influenced by either an alteration in the relative contributions of CI and CII- driven respiration in the electron transport chain (ETC) or an uncoupling of O_2 consumption from ATP synthesis (*i.e.*, non-phosphorylating respiration), was not

investigated in this previous work (Layec *et al.*, 2011; Layec *et al.*, 2012). Indeed, either or both of these scenarios of mitochondrial inefficiency could potentially play a role in the decreased mechanical efficiency and exercise intolerance observed in patients with COPD, but neither have been thoroughly investigated.

A shift in the contributions of CI and CII-driven respiration to peak respiration can affect mitochondrial efficiency, because electrons entering the ETC at CI, in the form of NADH, are at a higher energy state and subsequently result in the translocation of more protons than those entering at CII in the form of $FADH_2$. Thus, the $FADH_2$-derived electrons ultimately yield fewer ATP per O_2 consumed (*i.e.*, a lower P/O ratio) than their NADH-derived counterparts (Lee *et al.*, 1996). Therefore, a decreased contribution of CI-driven respiration in favor of a greater contribution of CII-driven respiration to overall respiration may result in a greater O_2 cost for a given workload, and potentially result in exercise intolerance. Furthermore, uncoupled O_2 consumption by the ETC occurs when hydrogen ions, pumped across the mitochondrial innermembrane in an O_2-dependent manner, bypass ATP synthase and pass down the proton gradient by other means (*e.g.*, uncoupling proteins), resulting in non-phosphorylating O_2 consumption or proton leak (Brand & Esteves, 2005). Currently, the extent to which uncoupled respiration is present in the skeletal muscle of patients with COPD is unclear. Indeed, the studies that have examined this facet of mitochondrial respiration in COPD have produced conflicting results reporting either exaggerated or unchanged uncoupling in COPD (Rabinovich *et al.*, 2007; Picard *et al.*, 2008b; Naimi *et al.*, 2011). Although these phenomena may contribute to the increased O_2 cost of exercise recognized in patients with COPD (Picard *et al.*, 2008; Bronstad *et al.*, 2012) and ultimately negatively impact exercise tolerance,

this hypothesis has not been extensively tested in this population.

Therefore, the purpose of this study was to determine if qualitative alterations in skeletal muscle mitochondrial respiration play a role in the exercise intolerance associated with COPD. Specifically, we hypothesized that, compared with healthy controls, patients with COPD would exhibit decreased mitochondrial efficiency with reduced utilization of CI-driven respiration relative to CII-driven respiration as well as increased uncoupling (State 2 respiration) and that this decreased mitochondrial efficiency may contribute to exercise intolerance during KE in the patients with COPD.

Methods

Subjects

Thirteen patients with moderate-to-severe COPD and thirteen age-matched, healthy controls were recruited for this study based on spirometric evidence of airway obstruction (FEV_1 <80% predicted, FEV/FVC<0.70), or the lack thereof (FEV_1 >80% predicted, FEV_1/FVC>0.70), respectively. These determinations were achieved by standard pulmonary function tests (Celli *et al.*, 2004). Thigh volume and muscle mass were assessed by a series of circumference and skin fold thickness measurements of the upper leg, as previously described (Layec *et al.*, 2014). Resting arterial O_2 (SaO_2) saturation was assessed with a pulse oximeter (Nellcor N-595, Pleasanton, CA, USA) placed on the tip of the middle finger following 5 minutes of seated rest. All patients and controls completed the study with the exception of one control who withdrew for reasons unrelated to the study. The Institutional Review Boards at the University of Utah and the Salt Lake City VA Medical Center approved all protocols employed in this study.

Accordingly, all subjects provided written informed consent before inclusion in this study.

Muscle Biopsy

Subjects reported to the laboratory for the muscle biopsy having refrained from vigorous exercise for 24 hours. A muscle sample of the vastus lateralis muscle was obtained by a percutaneous needle biopsy 15 cm proximal to the knee at a depth of 3.5 cm under sterile conditions (Richardson *et al.*, 2004). Immediately after the muscle sample (~150 mg) was taken from the leg, part of the sample (~30 mg) was immersed in ice-cold biopsy preservation fluid (BIOPS) for respiratory analyses (Pesta & Gnaiger, 2012), while the remaining sample was immediately frozen and stored at -80°C for later histological and biochemical analysis.

Mitochondrial Respiration and Histochemical Analyses

Muscle samples were prepared and permeabilized for mitochondrial respiration analysis as described by Pesta *et al.* (2012). Briefly, BIOPS-immersed fibers were carefully separated with fine-tip forceps and subsequently bathed in a BIOPS-based saponin solution (50µg saponin/ml BIOPS) for 30 minutes. Following saponin treatment, muscle fibers were rinsed twice in ice-cold mitochondrial respiration fluid (MIR05) for 10 minutes each rinse. After rinsing for a total of 20 minutes, fibers were blotted with a paper towel to measure the weight of each sample (2-4 mg).

Muscle fibers were then placed in the temperature-controlled respiration chamber (Oxytherm, Hansatech Instruments. Norfolk, UK) in 2 ml MIR05 solution and warmed to 37°C. After allowing the muscle fibers 10 minutes to equilibrate, mitochondrial

respiratory function was assessed using the protocol described in Table 3.1 to determine the peak CI-driven respiration (State 3:CI), the peak CII-driven respiration (State 3:CII), and the peak CI+CII-driven respiration (State 3:CI+CII). O_2 consumption not linked to phosphorylation (*i.e.*, State 2) was also assessed (Table 3.1). Importantly, all fibers included in this study exhibited evidence of mitochondrial membrane integrity (less than a 10% increase in respiration in response to cytochrome c). Initially, respiration data were examined in terms of O_2 flux per mg of tissue (wet weight) to obtain an indication of mitochondrial capacity per milligram of tissue. To further explore whether the mitochondria of patients with COPD exhibit qualitative changes in mitochondrial respiratory function, respiration was also examined in terms of flux control ratios (Pesta & Gnaiger, 2012). These internal ratios, which juxtapose one respiration state to another within an experimental run, provide insight into the qualitative function of the mitochondria in a mitochondrial content-independent manner (Pesta & Gnaiger, 2012). Muscle fiber type (Myosin Heavy Chain I and II) cross-sectional area, proportion and capillarity were assessed on frozen muscle samples by immunohistochemistry and morphometry using the methods described by Bloemberg *et al.* (2012).

Dynamic Single Leg Knee Extensor Exercise

Dynamic single leg KE exercise was performed on a custom-made KE ergometer (Andersen *et al.*, 1985; Richardson *et al.*, 1993). After undergoing several familiarization visits, subjects performed exercise protocols to determine both maximum KE work rate (*i.e.*, power) and endurance/exercise tolerance. Maximum KE work rate was determined with subject-specific incremental protocols (2-5 watts per minute) designed to reach a point of exhaustion within 8-12 minutes (Richardson *et al.*, 1995). KE endurance or

exercise tolerance was determined by having subjects perform KE at 80% of their maximum work rate until exhaustion (Rossman *et al.*, 2013). In both KE tests, subjects were instructed to maintain a cadence of 60 rpm until exhaustion. Exhaustion or task failure was defined as the inability to maintain a cadence of greater than 50 rpm.

Assessment of Physical Activity

Physical activity was assessed over the course of 7-10 days via accelerometry (Actigraph LLc, Pensacola Fl), which has recently been validated for use in COPD patients (Rabinovich *et al.*, 2013). These accelerometers, which assess physical activity in counts by summing the accelerations along three axes over the course of each minute, were worn by subjects during all waking hours, except when showering or swimming. Based on the recommendations of the manufacturer of the accelerometer, thresholds for sedentary, light, and moderate to vigorous activity were defined as <99, 100-1959, and 1952+ counts/min, respectively. Gait speed represents the usual walking speed (average of two trials) of the subjects over 10 meters (Schmid *et al.*, 2007).

Statistical Methods

Differences in respiration states were analyzed with two-way repeated measures ANOVA. Significant main effects or interactions were subsequently analyzed with Holm-Sidak post hoc test. Differences in subject characteristics between groups were made with independent samples t tests. Correlations between variables were assessed with Pearson product moment correlation. Data are represented as Mean ± SEM and α=0.05.

Results

Subject Characteristics

As represented in Table 3.2, patients and controls were of similar age and BMI. However, by design, spirometric indices of lung function were decreased in the patients with COPD ($P<0.05$). Nevertheless, resting O_2 saturation was similar between groups. KE maximum work rate was not different between groups, however, KE endurance was greatly reduced in the patients with COPD ($P<0.05$). Although all subjects underwent the biopsy procedure and completed all other testing, two of the patients with COPD failed to complete the KE protocols, for reasons unrelated to the study. Interestingly, there were no differences in fiber type or capillary density between the patients with COPD and the controls ($P>0.05$). Gait speed and physical activity, assessed by both accelerometry and steps per day, were lower in the patients with COPD than the healthy controls ($P<0.05$). Eleven of the thirteen patients with COPD were former smokers and reported an average time elapsed since quitting of 13 ± 3 years (range 2-28 years). Four of the controls were identified as former smokers and reported an average time elapsed since quitting of 39 ± 6 years (range of 22-60 years). One patient with COPD and none of the controls reported being current smokers. Aside from the medications subjects were free of any medications within the last 6 months.

Mitochondrial Respiration and Muscle Characteristics

In terms of respiration per mg of muscle, a significant interaction existed between subject group (*i.e.*, Control or COPD) and respiration states ($P<0.05$), such that the impact of COPD on respiration depended on the respiration state under consideration. Notably, State 3: CI (37.9 ± 2.7 and 23.9 ± 2.3 pMol/mg/s, $P<0.05$) and State 3:CI+CII

(46.5 ± 3.8 and 33.8 ± 3.8 pMol/mg/s, $P<0.05$) were both lower in patients with COPD ($P<0.05$), while State 3:CII (29.0 ± 2.3 and 26.8 ± 2.8 pMol/mg/s , $P>0.05$) was similar between groups. State 2 respiration, when expressed in terms of respiration per mg of muscle, was not significantly different between controls and patients (14.2 ± 1.5 and 9.0 ± 1.3 pMol/mg/s, $P=0.18$).

As wet weight muscle respiration indicated altered utilization of the CI and CII pathways in COPD, the relative contribution of each pathway in terms of percent of peak respiration during State 3:CI+CII (Figure 3.1B) was explored. Consistent with the data represented in terms of wet weight respiration (Figure 3.1A), a significant interaction between subject group and respiration state was observed ($P<0.05$). Further analysis revealed a diminished contribution of the CI pathway in COPD (83.7 ± 3.4 and 72.1 ± 2.4 % Peak, $P<0.05$) and further revealed an exaggerated contribution of the CII pathway (64.9± 3.2 and 79.5 ± 3.0 % Peak, $P<0.05$). State 2 respiration represented as a percentage of peak respiration was not different between groups (33.5 ± 4.1 and 32.3 ± 4.5 % Peak, $P<0.05$).

As it was hypothesized that patients would exhibit a greater reliance on CII-driven respiration than CI-driven respiration, additional analysis of the relative contribution of the CI- and CII-driven pathways with the CI/CII was performed. As illustrated in Figure 3.2A, CI/CII was significantly lower in patients with COPD compared to controls (0.82 ± 0.05 and 1.27± 0.05 respectively; $P<0.05$).

Mitochondrial Respiration, KE Endurance, and Lung Function

To explore the implications of this altered mitochondrial phenotype on exercise tolerance, the relationship between CI/CII and KE endurance (*i.e.*, time to exhaustion)

was examined (Figure 3.2B). Overall, CI/CII exhibited a significant relationship with KE endurance (r=0.43, P<0.05), which appears to have been driven largely by the moderate correlation within the patients (r=0.64, P<0.05), as no relationship was found when only the healthy controls were included in the correlation analysis (r=0.03; P=0.94). Lung function, as assessed by FEV_1, also exhibited a significant inverse relationship with CI/CII (r=0.55, P<0.05), with those subjects with a lower FEV_1 demonstrating a decreased utilization of the CI-driven pathway.

Discussion

The purpose of this study was to determine if qualitative alterations in skeletal muscle mitochondrial respiration are associated with exercise intolerance in patients with COPD. In terms of intrinsic mitochondrial function, patients with COPD exhibited a decreased reliance on CI-driven respiration and a greater reliance on the metabolically less-efficient CII-driven respiration, while uncoupled respiration was not different between groups. As detailed below, this study provides novel evidence that the exercise intolerance exhibited by patients with COPD is related to qualitative alterations in skeletal muscle mitochondrial respiratory function.

CI vs. CII-Driven Respiration in COPD

As already recognized, the exercise intolerance exhibited by patients with COPD is thought to be partly related to changes within the muscle that result in an increased O_2 cost of exercise (Richardson *et al.*, 2004; Medeiros *et al.*, 2014). Indeed, as has been reported previously (Picard *et al.*, 2008b; Naimi *et al.*, 2011; Bronstad *et al.*, 2012), in the current study muscle fibers from patients with COPD exhibited significantly reduced

rates of respiration per mg of muscle (Figure 3.1A). Certainly these reduced rates of O_2 consumption may impair aerobic capacity, especially when coupled with the reduced muscle mass sometimes exhibited by patients with COPD (Table 3.2). However, such attenuated respiration rates do not necessarily confer a qualitative change in the mitochondrial function that could explain the increased O_2-cost of exercise associated with COPD (Picard *et al.*, 2008b). Given that electrons entering the ETC at CII require more O_2 to resynthesize a given amount of ATP than electrons entering at CI (Lee *et al.*, 1996), we hypothesized that, patients with COPD would also exhibit an increased reliance on the less-efficient CII-driven respiration compared to the more-efficient CI-driven respiration. Indeed, such a pattern of altered respiration was evident in patients with COPD, as State 3:CI was significantly reduced in COPD compared to controls while State 3:CII was not (Figure 3.1). Thus, in agreement with previous research (Bronstad *et al.*, 2012), it appears that the reduction in State 3:CI+CII exhibited by patients with COPD in the current study was largely driven by diminished CI-driven respiration rather than CII-driven respiration. (Figure 3.1A). Consequently, when considered in relative terms, the current data indicate that CI-driven respiration makes up a lesser proportion of peak respiration and provides a smaller metabolic contribution than CII-driven respiration in patients with COPD than in healthy controls (Fig. 3.1B, 3.2A).

In light of decreased CI-driven respiration, the maintenance of CII-driven respiration per mg tissue in the patients with COPD likely prevented further loss of respiratory function in the muscle that would have occurred if CII-driven respiration was also decreased. However, it should be emphasized that in terms of ATP production, CI and CII-driven respiration are not equal. Even if CII-driven respiration was exaggerated

to the point that it to fully compensate for the diminished CI-driven respiration in terms of O_2 consumption, given the lower P/O ratio, such augmented CII-driven respiration would not fully offset the decrement in terms of ATP production (Lee *et al.*, 1996; Hinkle, 2005). Therefore, if patients with COPD do rely on this less-efficient metabolic pathway more than controls, the mitochondria of the patients would be obligated to respire more than their healthy counterparts to produce the same amount of ATP. This appears to have been the case for the patients studied by Richardson *et al.* (Richardson *et al.*, 2004) and Medeiros *et al.* (Medeiros *et al.*, 2014) who demanded greater amounts of O_2 to perform the same amount of work as their healthy counterparts. To date the P/O ratio from combined CI + CII driven respiration has not been fully explored in COPD. Previously, Puente-Maestu *et al.* (Puente-Maestu *et al.*, 2009) reported that the P/O ratio of mitochondria from patients with COPD was similar to healthy controls, but in this case the P/O ratio was only measured during CII-driven respiration. Based upon our data, the P/O ratio is more likely to be depressed when both CI and CII are stimulated simultaneously, as it may be the relative contribution of each pathway during physiological respiration and not necessarily the isolated function of each pathway that affects the P/O ratio in patients with COPD. While it is not clear if the qualitative alterations in mitochondrial respiration (*i.e.*, reduced CI/CII) observed in the current study were associated with a decreased P/O ratio, it is clear that this altered respiratory profile was related to exercise intolerance among the patients, as those who exhibited low CI/CII (*i.e.*, low contribution of CI with high contribution of CII) fatigued earlier than those patients who exhibited a greater CI/CII (Figure 3.2).

Currently, it is not clear why the mitochondria of patients with COPD exhibit

diminished CI-driven respiration compared to the mitochondria of healthy controls. Interestingly, Daussin *et al.* (Daussin *et al.*, 2008) reported a similar predominance of CII over CI-driven respiration in the mitochondria of sedentary individuals compared to those of endurance-trained individuals. In the current study, while both the patients and controls were relatively sedentary (Tudor-Locke & Bassett, 2004), the patients were less active than the controls (Table 3.2). Thus, it is possible that physical inactivity played a role in the diminished role of CI-driven respiration in COPD; however, it has previously been reported that altered mitochondrial respiration persists in patients with COPD even when matched for physical activity (Puente-Maestu *et al.*, 2009). In this regard, it should be noted that the observed differences in respiration in the current study existed despite there being no significant difference in muscle fiber type (Table 3.2). Additionally, it has recently been suggested that the mitochondrial DNA of patients with COPD is highly susceptible to oxidative stress (Puente-Maestu *et al.*, 2011; Konokhova *et al.*, 2013). Interestingly, CII represents the only complex of the ETC that is not encoded, at least in part, by mitochondrial DNA. Thus, the shift in ETC pathway to predominantly CII-driven respiration may potentially be the consequence of decreased CI respiration due to mitochondrial DNA damage. Certainly, further research is needed to determine the role of physical activity and mitochondrial DNA integrity in the diminished utilization of CI-driven respiration observed in patients with COPD.

Uncoupled Respiration in COPD

This study also tested the hypothesis that a component of the exercise intolerance typical of patients with COPD would be a consequence of an increase in uncoupled or non-phosphorylating O_2 consumption. Contrary to our hypothesis, State 2 respiration per

mg of muscle was not significantly augmented among patients with COPD (Figure 3.1A); in fact, it tended to be lower among patients with COPD. Additionally, there was no difference in State 2 respiration when expressed as a percentage of peak respiration (Figure 3.1B). Thus, this trend appears to be the consequence of reduced peak respiration rather than a qualitative change within the mitochondria. Thus, based on these data, it seems unlikely that exaggerated uncoupled respiration contributes to the exercise intolerance or the increased O_2-cost of exercise common to patients with COPD. Nevertheless, as noted earlier, evidence regarding uncoupled respiration in COPD is conflicting and somewhat complex. For example, Picard *et al.* (Picard *et al.*, 2008b) reported no significant difference in State 2 respiration between patients with COPD and controls, while Naimi *et al.* (Naimi *et al.*, 2011) reported that patients with COPD had significantly greater State 2 respiration than controls. As it has been demonstrated that cachexic patients with COPD are more likely to exhibit exaggerated uncoupling (Rabinovich *et al.*, 2007), the disagreement between findings, may to some extent reflect the heterogeneity of disease severity among patients.

Increased O_2 Cost of Contraction in an O_2-Limited Population

Based upon the current data, patients with COPD exhibit decreased mitochondrial efficiency in terms of CI/CII, but not in terms of uncoupling. When considered in isolation this decrease in mitochondrial efficiency may not appear to be a severe limitation in COPD, but when viewed in combination with other physiological alterations present in this patient population it becomes clear that this qualitative change in mitochondrial function has the potential to severely exacerbate exercise intolerance in COPD. First, it is worth noting that in patients with COPD O_2 delivery is often reduced

(Knower *et al.*, 2001; Dempsey *et al.*, 2006). For example, during exercise, particularly during large muscle mass exercise, patients with COPD exhibit varying degrees of pulmonary diffusion limitation that results in decreased blood O_2 saturation and content, which may compromise muscle O_2 delivery (Knower *et al.*, 2001). Additionally, patients with COPD exhibit an increased work of breathing for a given level of exercise, which may limit locomotor muscle O_2 delivery by redirecting a portion of a finite cardiac output, which may already exhibit a lower O_2 content, away from the exercising locomotor muscles and toward the respiratory muscles (Dempsey *et al.*, 2006). Thus, in patients with COPD, decreased mitochondrial efficiency may demand more O_2 from an already reduced supply. Second, the consequences of decreased mitochondrial efficiency may be magnified by the increased ATP cost of contraction that our group recently observed in patients with COPD (Layec *et al.*, 2011; Layec *et al.*, 2012). Specifically, it appears that not only do patients with COPD utilize a less efficient ETC pathway to synthesize ATP, but once synthesized, more ATP is required to elicit a contraction cycle, thereby exaggerating the O_2 cost of contraction even more. Third, given the decreased respiratory capacity per mg of muscle reported in this study (Figure 3.1A) and other studies (Picard *et al.*, 2008b; Naimi *et al.*, 2011; Bronstad *et al.*, 2012), the ability of patients with COPD to meet the increased demand for O_2 consumption due to altered mitochondrial efficiency is potentially hindered.

Together the multiple alterations in O_2 supply by the cardiopulmonary system and O_2 demand by the exercising muscle in patients with COPD likely converge to create a supply-demand mismatch where the exercising muscle demands more O_2 to perform a given amount work and this is compounded by a diminished O_2 supply. While it is

unclear whether all of these factors converged in the current patients with COPD, it is evident that the documented reduction in mitochondrial efficiency was related to exercise intolerance (Figure 3.2B), thus implying that this phenomenon may play a role in the limited exercise capacity exhibited by these patients.

Conclusions

Qualitative alterations in skeletal muscle mitochondrial function that emphasize the relatively less efficient CII-driven respiration over the more-efficient CI-driven respiration appear to contribute to decreased muscle function and exercise intolerance in patients with COPD. As patients with COPD often experience compromised O_2 supply, such an increase in metabolic demand may yield deleterious effects even during the relatively modest physical demands of performing simple activities of daily living.

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Table 3.1: Mitochondrial Respiration Protocol: Description of the protocol used to assess mitochondrial respiratory function, and the site of action of each chemical introduced to the preparation (+ Substrate; -Inhibitor), and the respiration state associated with each step. Note that each step was approximately 3 minutes in duration.

Step #	Chemical Name (concentration)	Major Site of Action	Respiration State
1	Malate (2mM), Glutamate (10mM)	+Complex I (CI)	State 2
2	ADP (5mM)	+Complex V (CV)	State 3: CI
3	Succinate (10mM)	+Complex II (CII)	State 3: CI+CII
4	Cytochrome C (10μM)	Test of mitochondrial membrane integrity	
5	Rotenone (0.5μM)	-CI	State 3: CII

Adenosine Diphosphate, ADP

Table 3.2: Subject Characteristics

	Controls	COPD	*P*
Subjects (female, male)	12 (2f, 10m)	13 (3f, 10m)	
Age (years)	69± 2	66 ± 2	0.36
BMI (kg/m^2)	26±1	27 ± 2	0.73
Lung Function			
FVC (Liters)	4.8 ± 0.3	3.2 ± 0.2*	<0.001
FEV_1 (Liters)	3.5± 0.2	1.7 ± 0.2 *	<0.001
FEV_1 (% Predicted)	119 ± 6	55 ± 5 *	<0.001
FEV_1/FVC (%)	79 ± 3	51 ± 4 *	<0.001
Resting SaO_2 (%)	95±1	94±1	0.57
Physical Activity and Function			
Knee Extensor Max (watts)	29 ±4	24 ± 4	0.38
Knee Extensor Endurance (mins)	14 ± 3	8 ± 1 *	0.04
Gait Speed (m/s)	1.38 ± 0.03	1.12 ± 0.08*	0.007
Steps Per Day	5387±648	3067±250*	0.004
Sedentary Physical Activity (mins/day)	1245±23	1321±15*	0.01
Light Physical Activity (mins/day)	160±16	111±10	0.14
Moderate-to-Vigorous Physical Activity (mins/day)	25 ± 4	8 ± 2*	0.003
Muscle Characteristics			
Quadriceps Muscle Mass (kg)	1.8 ± 0.1	1.4 ±0.1	0.06
Type 1 Fibers (%)	40±3	40±5	0.61
Type 2 Fibers (%)	60±3	60±5	0.61
NCAF	2.7±0.05	2.6±0.05	0.78
Medication History			
Inhaled β-Agonist/Cholinergic Antagoinst (n)	0	7*	<0.001
Corticosteroid (n)	0	0	1.00
Statin-Type Drugs (n)	2	2	0.93
Muscle Relaxant (n)	0	2	0.14

Body Mass Index, BMI; Forced Vital Capacity, FVC; Forced Expiratory Volume in One Second, FEV_1; Arterial Oxygen Saturation, SaO_2; Number of Capillaries Around a Fiber, NCAF, Medications listed are those taken over the past 6 months

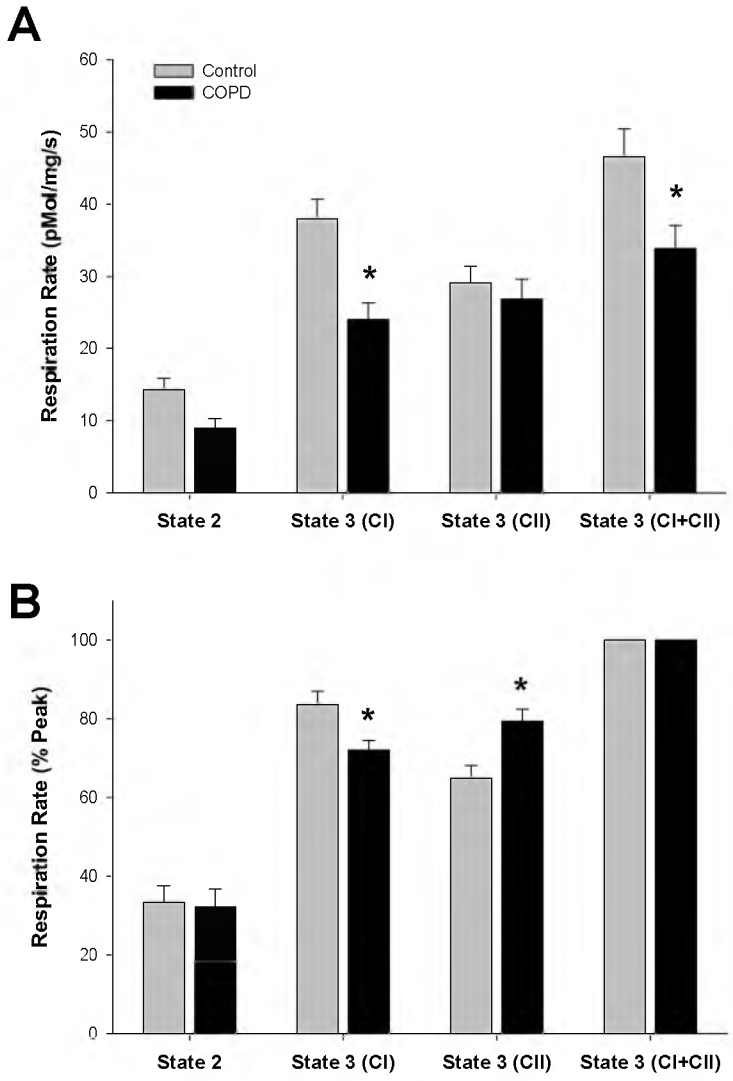

Figure 3.1: Mitochondrial respiration of vastus lateralis muscle from patients with COPD and healthy controls. A.) Mitochondrial O_2 flux per mg of muscle. **B.)** Mitochondrial respiration normalized to peak respiration observed during State 3 Respiration of Complex I and Complex II combined * Significantly different than healthy control.

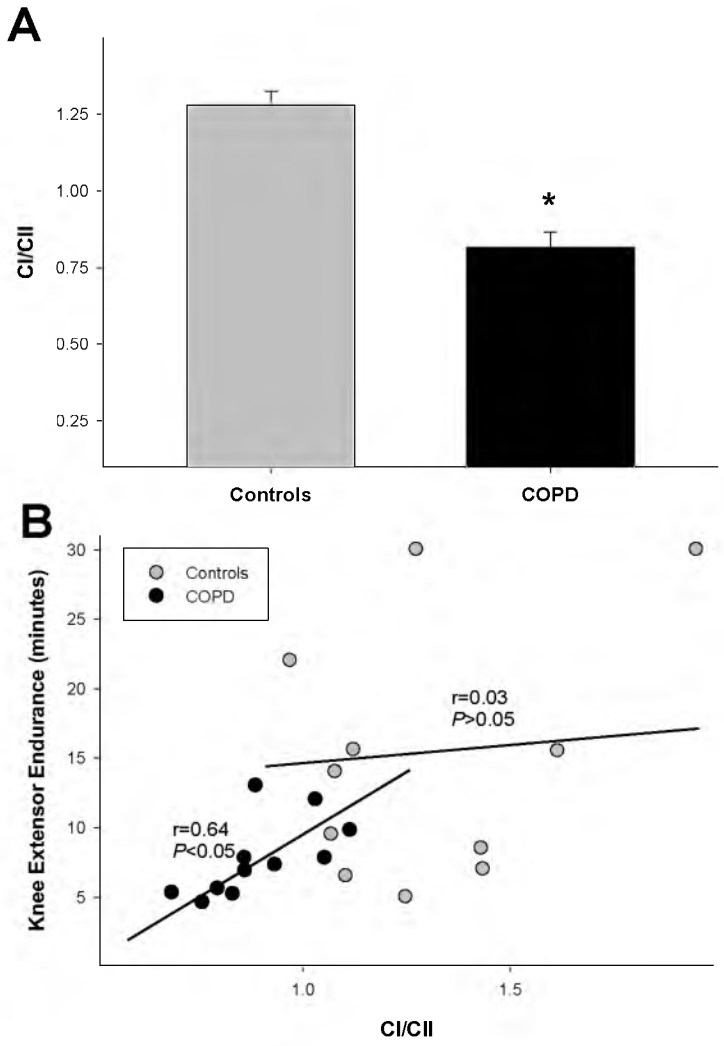

Figure 3.2: Relative contributions of Complex I and Complex II-driven respiration and knee extensor endurance A.) Ratio of State 3:CI to State 3:CII (*CI/CII*). **B.)** Relationship between CI/CII and knee extensor endurance. Note the significant relationship in the patients with COPD and lack of such a relationship in healthy controls. * Significantly different than healthy controls.

CHAPTER 4

SYMMORPHOSIS AND SKELETAL MUSCLE VO_{2MAX}: *IN VIVO* AND *IN VITRO* MEASURES REVEAL DIFFERING CONSTRAINTS IN THE EXERCISE TRAINED AND UNTRAINED HUMAN

Abstract

The hypothesis of symmorphosis postulates a quantitative match of structural and functional parameters within a defined system, but how this relates to oxygen (O_2) supply and demand in determining maximal skeletal muscle O_2 consumption (VO_{2max}) in humans is unclear. Therefore, skeletal muscle VO_{2max} was assessed *in vitro* ($_{Mito}VO_{2max}$, vastus lateralis permeabilized fibers) and *in vivo* during single leg knee extensor (KE) exercise ($_{KE}VO_{2max}$, direct Fick with femoral arterial blood flow (Doppler ultrasound) and arterial and venous blood samples) in 10 endurance-exercise trained and 10 untrained young males. Whole-body cycling VO_{2max} ($_{Body}VO_{2max}$, indirect calorimetry) was also measured. In the untrained subjects O_2 delivery (462±37 ml/kg/min) during KE exercise exceeded $_{Mito}VO_{2max}$ (364±16 ml/kg/min, $P<0.05$), which was not significantly different from $_{KE}VO_{2max}$ (340±22 ml/kg/min, $P>0.05$). Conversely, in the trained subjects O_2 delivery during KE exercise (557±35 ml/kg/min) was significantly less than $_{Mito}VO_{2max}$ (743±35 ml/kg/min, $P<0.05$) which, in turn, was significantly greater than $_{KE}VO_{2max}$ (458±24 ml/kg/min). Additionally, while $_{Mito}VO_{2max}$ in the untrained subjects was related to $_{KE}VO_2max$ ($r=0.69$, $P<0.05$) and $_{Body}VO_{2max}$ ($r=0.91$, $P<0.05$), these variables were entirely unrelated in the trained subjects. Together these *in vivo* and *in vitro* measures reveal clearly differing determinants of VO_{2max} in untrained and trained humans. Specifically, in the untrained, there is evidence against symmorphosis, with VO_{2max} being determined by mitochondrial O_2 demand. While, in contrast, trained subjects exhibit an exercise training-induced mitochondrial reserve that results in skeletal muscle VO_{2max}, *in vivo*, being O_2 supply limited, also not fitting the postulate of symmorphosis.

Introduction

VO_{2max}, assessed during an incremental graded exercise test, is a strong predictor of mortality in both health and disease (Lee *et al.*, 1999; Laukkanen *et al.*, 2001; Kurl *et al.*, 2003). However, despite the well-known predictive value of VO_{2max}, it still remains unclear which step, if any, along O_2 cascade limits VO_{2max} (Betik & Hepple, 2008). Based on a derivation of the Fick principle, VO_2 is the product of muscle O_2 delivery (QO_2) and mitochondrial O_2 extraction (VO_2=QO_2 x O_2 extraction) (Richardson, 2003), and thus the steps of the O_2 cascade that potentially determine VO_{2max} can be generalized into two components: O_2 supply to the skeletal muscle mitochondria by the cardiopulmonary system and O_2 demand by the skeletal muscle mitochondria. Supported by the tight relationship between mitochondrial volume and VO_{2max} across a broad range of mammalian species, proponents of the theory of symmorphosis have suggested that all steps of the O_2 cascade are built in strict proportion to the task required of them at VO_{2max}. Thus, neither the capacity for O_2 supply nor the capacity for O_2 demand would be in excess or limiting at VO_{2max} (Hoppeler & Weibel, 1998). While this concept applies well when considering variation in VO_{2max} across many species, how well symmorphosis pertains to humans is unclear as evidence and speculation from studies in humans consistently implicate distinct factors that may limit VO_{2max} (Hoppeler & Weibel, 1998; Wagner, 2000; Levine, 2008).

While most agree that a rate-limiting step likely exists along the O_2 cascade in humans, it is unclear whether the bottleneck is in the O_2 supply to the mitochondria (Knight *et al.*, 1992; Levine, 2008; Boushel *et al.*, 2011) or O_2 demand by the mitochondria (Roca *et al.*, 1992; Cardus *et al.*, 1998), and, subsequently, whether the

capacity of either of these processes are actually in excess at VO_{2max}. In a study of similar design to the current work, Boushel *et al.* (4) attempted to shed light on this issue by comparing $_{Mito}VO_{2max}$ in permeabilized muscle fibers to the mass-specific VO_{2max} of the lower limb during cycling exercise. As mass-specific cycling VO_{2max} did not achieve the level of $_{Mito}VO_{2max}$, it was concluded that cycling VO_{2max} was limited by O_2 supply. However, utilization of the cycling exercise paradigm leaves uncertainty as to the proportion of the lower-limb muscle mass that was actually maximally recruited in this study (Lollgen *et al.*, 1980; Green & Patla, 1992). Therefore, it is unclear whether the low mass-specific cycling VO_{2max} was due to limited O_2 delivery, as suggested, or simply due to an overestimation of the amount of muscle mass that was maximally recruited during cycling. As physical activity is recognized to greatly increase mitochondrial capacity, an additional, potentially confounding, factor that may have yielded conflicting results in this area is the training status of the subjects. Indeed, it seems possible that endurance-training, which consistently results in a relatively small increase in VO_{2max} compared to the large documented increase in mitochondrial capacity (Gollnick *et al.*, 1973), may result in a mitochondrial reserve capacity that shifts the bottleneck at VO_{2max} away from the mitochondria toward processes related to O_2 supply.

Therefore, with the concept of symmorphosis in mind, the purpose of this study was to determine the role of O_2 supply and demand in determining VO_{2max} in trained and untrained humans using an exercise model with well-defined muscle recruitment (Richardson *et al.*, 1998). Specifically, $_{Mito}VO_{2max}$ of permeabilized muscle fibers, from the vastus lateralis, were compared to maximum rates of O_2 delivery and VO_2, assessed by the direct Fick method, during maximal KE exercise in trained and untrained subjects.

We hypothesized that $_{KE}VO_{2max}$ of the untrained subjects would reach $_{Mito}VO_{2max}$, implicating mitochondrial O_2 demand as the limitation to VO_{2max} in these subjects. Conversely, we further hypothesized that, as a result of endurance training-induced mitochondrial biogenesis, trained subjects would exhibit a disproportionately high $_{Mito}VO_{2max}$, which would not be reached at $_{KE}VO_{2max}$, implicating O_2 supply as a determinant of VO_{2max} in these subjects. If these hypotheses were proven to be correct, this study would shed additional light on the concept of symmorphosis, as it relates to VO_{2max} in humans, revealing a lack this phenomenon in both untrained and trained subjects, but due to very differing constraints.

Methods

Subjects

Following approval by the Institutional Review Board at the University of Utah and the Salt Lake City Veterans' Hospital, 10 young, healthy untrained males and 10 young, healthy, endurance-trained male subjects were recruited for this study and provided informed, written consent. The untrained subjects were selected based upon a self-reported lack of moderate-to-vigorous physical activity over the past 2 years, while the trained subjects reported regularly participating in moderate-to-vigorous physical activity and endurance training (*e.g.*, cycling, running, skiing) over the past 2 years.

Muscle O_2 Delivery and Muscle VO_2max *in vivo*

Familiarization Sessions

Prior to the main experiment, subjects underwent multiple familiarization visits to become accustomed to performing KE exercise on a custom built knee extension

ergometer and cycling exercise on a commercially available cycle ergometer (Lode, Netherlands). Once familiar with the exercise modalities, the maximum work rate (WR_{max}) a subject could sustain for 1 minute at the end of maximal graded cycle and KE exercise test was assessed. Specifically, for both cycling and KE exercise, subjects were given a 2-3 minute warm-up which was followed by an increment in WR (KE: 5 W, Cycling: 25 W) each minute until task failure (*i.e.* inability to maintain 60 rpm during KE exercise or falling to 50 rpm from a >60 rpm from a self-selected cadence during cycling). In order to ensure a test duration of 8-12 minutes, the starting workload for both exercise modalities was tailored to each subject's ability. Additionally, $_{Body}VO_{2max}$ was assessed during the cycling WRmax test utilizing an indirect calorimetry system (Parvomedics, UT, USA), and was defined as the highest rate of O_2 consumption averaged over 30 seconds prior to cessation of exercise (Garten *et al.*, 2014).

Experimental Protocol

On the day of the exercise portion of the experiment, subjects performed KE exercise at 25, 50, 75, 90, 100% of WR_{max} for 2-3 minutes each (Richardson *et al.*, 1995), during which muscle VO_2 of the leg was assessed by the direct Fick method. Specifically, muscle VO_2 was calculated as the product of femoral blood flow and the arterial-venous O_2 difference (*VO_2= Femoral Blood Flow * a-v O_2 difference*). Femoral blood flow was assessed via Doppler ultrasound of the common femoral artery (CFA), proximal to the exercising quadriceps femoris, while arterial and venous O_2 content was assessed by a blood gas analyzer (GEM 4000, Instrumentation Laboratories, Bedford, MA, USA) on blood drawn through catheters placed directly in the CFA and common femoral vein (CFV), as described below. Subsequently, QO_2 to the muscle was

calculated as the product of femoral blood flow and arterial O_2 content. Heart rate was assessed at rest and throughout the test with a standard three-lead ECG. Femoral artery blood pressure was measured continuously at rest and during exercise with an indwelling catheter placed in the CFA with a pressure transducer set at the level of the catheter. Subsequently, heart rate, blood pressure and power output data were recorded with a data acquisition system (Biopac, Goleta, Ca).

Doppler Ultrasound Assessments

Measurements of CFA blood velocity and artery diameter, 2-3 cm proximal to the superficial/deep bifurcation, were taken using a Logiq 7 ultrasound Doppler system in duplex mode (General Electric Medical Systems, Milwaukee, WI) equipped with a linear array transducer function at an imaging frequency of 14 MHz. CFA blood velocity was assessed with the same probe with a Doppler frequency of 5 MHz operated in the high-pulsed repetition frequency mode (2-25mHz), as described previously (Barrett-O'Keefe *et al.*, 2013). Blood velocity was assessed with an insonation angle of no more than 60° (Harris *et al.*, 2010) while the sample size was maximized and centered according to vessel size and position in real time. All ultrasound data were calculated using Logiq 7 software. Ultimately, femoral blood flow was calculated with the following equation:

$$\textit{Femoral Blood Flow} = [\textit{mean blood velocity} * \pi(\textit{vessel diameter}/2)^2 * 60].$$

Blood Analysis

Three milliliters of blood were drawn at rest and during the last minute of each exercise stage through catheters placed in the CFA and CFV. Approximately 1 ml of blood was analyzed for arterial and venous total hemoglobin (tHb) content, hemoglobin

saturation (HbO_2), and PO_2 using a GEM 4000 blood gas analyzer and cooximeter (Instrumentation Laboratories, Bedford, Ma). Arterial and venous blood O_2 content was calculated with the following equation: *Blood O_2 Content (in ml/dl) = [1.39(tHb) * (HbO_2 saturation/100)] + (0.003 * PO_2)]*. The remainder of blood drawn was taken and preserved for further analysis.

Determination of Leg Muscle Mass

Muscle volume of the lower limb was assessed by multiple measurements of thigh and leg circumference and skin fold thickness as described by Layec *et al.* (2014), who documented that this simple estimate of muscle volume demonstrates a very strong correlation with volume measured by MRI across a broad spectrum of subjects (r^2=0.89-0.98). Muscle mass of the thigh and leg were then determined by assuming a muscle density of 1.049kg/L (Kemp *et al.*, 2015). Finally, the mass of the quadriceps femoris was subsequently calculated as described by Jones and Pearson (1969), while applying the correction factor recommended by Radegran *et al.* (1999).

Determination of Mitochondrial VO_2max *In Vitro*

Muscle Biopsy

Subjects reported to the laboratory for a muscle biopsy (approximately 1 week before or after the exercise/catheter portion of the study) in a fasted state and having refrained from vigorous exercise for 24 hours. Muscle samples of the vastus lateralis were obtained by a percutaneous needle biopsy 15 cm proximal to the knee at a depth of ~3.5 cm under sterile conditions (Richardson *et al.*, 2004). Immediately after the muscle sample (~150 mg) was removed from the leg, ~20% of the sample was immersed in ice-

cold biopsy preservation fluid (BIOPS) for respiratory analysis (Pesta & Gnaiger, 2012), while the other portion was snap frozen and stored at -80°C for histological and biochemical analyses.

Mitochondrial Respiration

Muscle samples were prepared and permeabilized as described by Pesta *et al.* (2012). Briefly, BIOPS-immersed fibers were carefully separated with fine-tip forceps and subsequently bathed in a BIOPS-based saponin solution (50µg saponin/ml BIOPS) for 30 minutes. Following saponin treatment, muscle fibers were rinsed twice in ice-cold mitochondrial respiration fluid (MIR05) for 10 minutes each rinse. The wet weight of each muscle sample (~3-4mg) was then assessed on a calibrated scale after excess fluid had been removed from the sample by dabbing the muscle several times with a paper towel.

Muscle fibers were then placed in the temperature-controlled respiration chamber (Oxytherm, Hansatech Instruments. Norfolk, UK) in 2 ml MIR05 solution and warmed to 37°C. After allowing the muscle 10 minutes to equilibrate, mitochondrial respiration was assessed in duplicate as described below. State 2 respiration was assessed with malate (2mM) + glutamate (10mM), followed by State 3:CI respiration with ADP (5mM). Subsequently, succinate (10mM) was added to assess State 3:CI+CII respiration, which represented $_{Mito}VO_{2max}$. Finally, cytochrome C (10uM) was added to the bath to verify membrane integrity as recommended by Pesta *et al.* (Pesta & Gnaiger, 2012). Each respiration state or step lasted as long as required to produce a steady state respiration rate, which was approximately 3 minutes in most cases. Background respiration inherent to the experimental setup was measured and taken into account when

assessing overall mitochondrial respiration. As the respirometer offered the greatest temperature stability at 37°C, but exercise generally results in an increase in muscle temperature from 37°C to 38+°C (Saltin *et al.*, 1968; Kenny *et al.*, 2003), respiration rates were obtained with the muscle sample at 37°C and then mathematically adjusted, based on a Q10 of 2 (multiplication factor for O_2 consumption at a 10°C difference), to yield predicted values at 38°C (Rasmussen *et al.*, 2001; Boushel *et al.*, 2011). The respiration data for each of the two separate muscle fiber samples were then averaged.

Statistical Analyses

Differences in O_2 delivery, O_2 consumption, and O_2 extraction during KE exercise were assessed with two-way, repeated measures ANOVA, which, when significant, was followed by a Tukey's post hoc test (SPSS version 17. Chicago, Il). Differences in variables between groups, such as subject characteristics and maximum values, were tested with an independent samples T-test. Correlations between variables were assessed with Pearson product-moment correlations. Alpha was set at 0.05 *a priori*.

Results

Subject Characteristics

Ten young, healthy, untrained and trained male subjects were successfully recruited and completed this study. As documented in Table 4.1, the subjects in both groups were well matched, except, by experimental design, as a consequence of exercise training, there was a clear difference in exercise capacity assessed by $_{Body}VO_{2max}$. Specifically, $_{Body}VO_{2max}$ in the untrained ranged from 31-44 ml/kg/min and the trained ranging from 54-66 ml/kg/min. This difference in $_{Body}VO_{2max}$ was also apparent in

absolute terms (*i.e.*, $_{Body}VO_{2max}$ in L/min) and reflected in both cycle and KE exercise WR_{max}, for each of which the untrained subjects were significantly lower than the trained subjects. Interestingly, although not achieving statistical significance, morphometric analyses of the vastus lateralis revealed a tendency for the untrained subjects to have a lower % of Type I skeletal muscle fibers and a greater % of Type II than the trained subjects, and no difference in the number of capillaries around a fiber (NCAF).

Response to KE Exercise

As illustrated in Figure 4.1A, mass-specific VO_2 during KE exercise increased with each increase in WR ($P<0.05$) until WR_{max}, except for the change in VO_2 from 90 to 100 % of KE exercise WR_{max} which did not achieve significance in either the untrained or trained subjects. As expected, the trained subjects achieved significantly greater rates of muscle O_2 consumption compared to untrained subjects during KE exercise, both in absolute (untrained: 641±41 ml/min; trained: 858±55 ml/min) or relative terms (Figure 4.1A). Femoral artery blood flow (Figure 4.1B) followed a very similar pattern to VO_2, with clear increments at each WR, except from 90-100% of KE exercise WR_{max}, and ultimately attaining significantly greater values at KE exercise WR_{max} in the trained subjects compared to the untrained subjects. a-v O_2 difference (Figure 4.1C) did not exhibit an increase with each KE exercise WR, in fact, in the untrained subjects extraction failed to increase beyond that achieved at 50% of WR_{max}. Interestingly, although also failing to increase O_2 extraction with each workload, the trained subjects exhibited a greater propensity for extracting O_2 by increasing a-v O_2 difference at WR_{max} beyond that achieved at 75%WR_{max}. Ultimately, a-v O_2 difference was significantly greater at KE exercise WR_{max} in the trained subjects compared to the untrained subjects.

Utilization of Mitochondrial Capacity during KE Exercise and Whole-Body Exercise

As is commonly observed with endurance training, muscle samples from endurance-trained subjects exhibited a significantly greater $_{Mito}VO_{2max}$ than untrained subjects (743 ± 35 and 364±16 ml/kg/min, respectively; Figure 4.2A). As illustrated in Figure 4.2, in untrained subjects $_{KE}VO_{2max}$ (340±21 ml/kg/min) was not significantly different than $_{Mito}VO_{2max}$ ($P>0.05$), while maximum O_2 delivery during KE exercise ($_{KE}QO_{2max}$, 462±37 ml/kg/min) exceeded $_{Mito}VO_{2max}$ ($P<0.05$). In contrast, in the trained subjects $_{KE}QO_{2max}$ (557±35 ml/kg/min) and subsequently $_{KE}VO_{2max}$ (458±24 ml/kg/min) were both significantly lower than $_{Mito}VO_{2max}$ ($P<0.05$). Of note, an increase in $_{Mito}VO_{2max}$ was associated with a proportionate increase in $_{KE}VO_{2}max$ among the untrained subjects ($r=0.69$, $P<0.05$), but not in the trained subjects ($r=0.01$, $P=0.74$, Figure 4.3). Interestingly, as illustrated in Figure 4.4, the untrained subjects exhibited a strong positive relationship between $_{Mito}VO_{2max}$ and $_{Body}VO_{2max}$, while, again in contrast to the untrained subjects, the trained exhibited no relationship between $_{Mito}VO_{2max}$ and $_{Body}VO_{2max}$ ($r=0.12$, $P=0.74$).

Discussion

The extent to which VO_{2max} represents the maximal capacity of each step of the O_2 cascade and therefore conforms to the postulate of symmorphosis, or which step, if any, limits VO_{2max} is keenly debated. This study, utilized a unique combination of *in vitro* and *in vivo* measures of respiratory capacity in both exercise-trained and untrained subjects to better elucidate the determinants of VO_{2max} in humans. Together these measures reveal clearly differing determinants of VO_{2max} in untrained and trained

humans. Specifically, in the untrained, there is evidence against symmorphosis, with VO_{2max} being determined by mitochondrial O_2 demand. While, in contrast, trained subjects, who also fail to fit the postulate of symmorphosis, exhibit an exercise training-induced mitochondrial reserve that results in skeletal muscle VO_{2max} *in vivo* being O_2 supply limited. Supportive of these differing determinants of VO_{2max}, in untrained subjects, pulmonary VO_{2max} assessed during more traditional whole-body cycling exercise revealed a strong and significant relationship to muscle mitochondrial VO_{2max} measured *in vitro*, while these two variables were entirely unrelated in the trained subjects. Thus, as VO_{2max} was suppressed by discrete, albeit different, portions of the O_2 cascade in exercise-trained and untrained humans, it appears that, in contrast to the premise of symmorphosis, the capacity of each component of the O_2 cascade is not expressed in strict proportion toVO_{2max}.

Utilization of Mitochondrial Capacity during KE Exercise

In order to gain insight into the extent to which untrained and trained subjects utilize their entire mitochondrial capacity during exercise, we compared $_{Mito}VO_{2max}$ to $_{KE}VO_{2max}$. KE exercise is a small-muscle-mass model that isolates an easily quantifiable muscle mass (Richardson *et al.*, 1998) and provides an experimental paradigm that lends itself to the assessment of blood flow and blood samples across the exercising muscle group, even at maximal exercise. Given the minimal strain that KE exercise places on central factors, such as the lungs and heart, we anticipated that if subjects were to reach $_{Mito}VO_{2max}$, *in vivo*, during some form of exercise, it would likely be KE exercise when pulmonary O_2 diffusion and cardiac output are not likely to be limiting. As illustrated in Figure 4.1, KE exercise yielded high maximal rates of O_2 consumption, which were

greater in the trained compared to the untrained subjects. As hypothesized, during KE exercise the untrained subjects were able to exercise to the point where $_{KE}VO_{2max}$ reached $_{Mito}VO_{2max}$ (Figure 4.2B). Indeed, these untrained subjects utilized 94±4% of their mitochondrial respiratory capacity during maximal KE exercise. Furthermore, within these untrained subjects $_{KE}VO_{2max}$ and $_{Mito}VO_{2max}$ were well related ($r=0.69$, $P<0.05$, Figure 4.3). Thus, in combination, these data imply that mitochondrial respiratory capacity limits $_{KE}VO_{2max}$ in untrained subjects. The trained subjects achieved significantly greater rates of O_2 consumption during KE exercise than the untrained subjects, but the increase in $_{KE}VO_{2max}$ was not proportionate to the large increase in $_{Mito}VO_{2max}$, which was nearly double that of the untrained subjects. Consequently, the trained subjects were only able to utilize 63±4% of their far greater $_{Mito}VO_{2max}$ during maximal KE exercise (Figure 4.2B), suggesting that mitochondrial capacity was likely not limiting VO_{2max} in these subjects.

Based on the observation that QO_{2max} failed to reach similar O_2 flux rates as $_{Mito}VO_{2max}$ among trained subjects (Figure 4.2A), it is likely that limited O_2 delivery prevented the trained subjects from reaching $_{Mito}VO_2max$ during maximal KE exercise. This is further supported by the lack of a relationship between $_{Mito}VO_{2max}$ and $_{KE}VO_{2max}$ in the trained subjects and contrasts starkly with the significant relationship exhibited by untrained subjects (Figure 4.3). Specifically, if the mitochondria were a limiting factor in $_{KE}VO_{2max}$ it would be reasonable to expect that across subjects with differing levels of $_{Mito}VO_{2max}$ there would be a concomitant change in $_{KE}VO_{2max}$, but this was apparent only in the untrained subjects. In the trained subjects there was no such relationship, implying that for trained subjects a factor upstream from the mitochondria,

in the O_2 cascade, is limiting $_{KE}VO_{2max}$.

Thus, it appears that mitochondrial respiratory capacity limits $_{KE}VO_{2max}$ in untrained subjects, while in trained subjects there is evidence of a mitochondrial reserve capacity. These differing constraints to VO_{2max} in trained and untrained humans during KE exercise may help to explain the contradictory findings on the effects of hyperoxia on $_{KE}VO_{2max}$ that have been reported (Pedersen *et al.*, 1999; Richardson *et al.*, 1999). For example, with untrained subjects, Pedersen *et al.* (1999) found that augmenting O_2 delivery by way of hyperoxia had no effect on $_{KE}VO_{2max}$. Based on the current data, it makes intuitive sense that there was no effect as the untrained subjects appear to already be operating at $_{Mito}VO_{2max}$ in normoxic conditions. In contrast, when studying the effects of hyperoxia on $_{KE}VO_{2max}$ in well-trained cyclists, Richardson *et al.* (1999) reported that hyperoxia significantly augmented $_{KE}VO_{2}$max by 17%. This finding is also in agreement with the current data, which indicate that trained subjects, who exhibit a significant mitochondrial reserve capacity under normoxic conditions, have the additional mitochondrial in place to consume the extra O_2 available in hyperoxia.

The current relationship between $_{Mito}VO_{2max}$ and $_{KE}VO_{2max}$ in the untrained subjects is supported by data from Rasmussen *et al.* (Rasmussen *et al.*, 2001) and Blomstrand *et al.* (Blomstrand *et al.*, 1997) who reported that *in vitro* measures of skeletal muscle mitochondrial capacity parallel $_{KE}VO_{2max}$. Of note, in addition to reporting that several indices of muscle metabolism exhibited good agreement with $_{KE}VO_{2max}$, Rasmussen *et al.* (Rasmussen *et al.*, 2001), somewhat surprisingly, also found that $_{Mito}VO_{2max}$, assessed in isolated mitochondria, was lower than $_{KE}VO_{2max}$. This observation, which disagrees with the current, more intuitive findings, is unlikely to say

the least. This physiologically improbable scenario, may have been the result of altered mitochondrial function, inherent to the isolated mitochondria technique (Picard *et al.*, 2011), or inflated blood flow measurements, attained by the thermodilution technique, that would result in an overestimation of $_{KE}VO_{2max}$.

Utilization of Mitochondrial Capacity during Whole-body Exercise

Recognizing the atypical nature of KE exercise and the minimal strain placed upon central factors, such as the lungs and heart, with this exercise modality, $_{Body}VO_{2max}$ during traditional cycle exercise was also assessed and compared with $_{Mito}VO_{2max}$ in these trained and untrained subjects. In support of the findings with KE exercise, as illustrated in Figure 4.4, the relationship between $_{Mito}VO_{2max}$ and $_{Body}VO_{2max}$, again very clearly, reveals that training status affects the relationship between respiratory capacity measured *in vitro* and whole body metabolic capacity during cycling. Indeed, as was the case for $_{KE}VO_{2max}$, but even more clearly, $_{Mito}VO_{2max}$ appears to have a significant influence on $_{Body}VO_{2max}$ in the untrained subjects during cycling. Specifically, across the untrained subjects, there was a strong positive relationship, such that each increase in $_{Mito}VO_{2max}$ was mirrored by a proportional increase in $_{Body}VO_{2max}$. Thus, in addition to the current KE exercise data and previous studies (Roca *et al.*, 1992; Cardus *et al.*, 1998) these findings support the conclusion that during exercise untrained subjects are limited by mitochondrial O_2 demand.

In contrast to the untrained, as was the case with KE exercise, among the trained subjects, an increase in $_{Mito}VO_{2max}$ did not result in an increase in $_{Body}VO_{2max}$. In agreement with prior studies utilizing cycling exercise (Roca *et al.*, 1992; Knight *et al.*, 1993), this finding suggests that, when exercise trained, mitochondrial capacity does not

dictate or limit $_{Body}VO_{2max}$. Indeed, increasing QO_2 has an effect on cycling VO_{2max} in trained subjects, who exhibit an 8% increase in cycling VO_{2max} when breathing 100% O_2 (Knight *et al.*, 1993). However, as already recognized, O_2 supply does not appear to be limiting in untrained subjects, as increasing, or even mildly decreasing O_2 supply has no effect on cycling VO_{2max} (Roca *et al.*, 1992; Cardus *et al.*, 1998). This divergence in the determination of VO_{2max} in untrained and trained subjects has also been observed in longitudinal studies of subjects before and after endurance training, supporting the idea that training is at the root of the differing constraints to VO_{2max} (Roca *et al.*, 1992).

Interestingly, as already acknowledged, in disagreement with the current study, Boushel *et al.* (Boushel *et al.*, 2011) reported that cycling VO_{2max} did not reach the level of $_{Mito}VO_{2max}$ in untrained subjects and thus there was an apparent mitochondrial excess during maximal cycling exercise. The discrepancy between the current study and that of Boushel *et al.* (Boushel *et al.*, 2011) could be related to differences in the aerobic fitness of the untrained subjects (*i.e.*, $_{Body}VO_{2max}$), which was not reported by Boushel *et al.*, or alternatively, may be related to an overestimation of the active muscle mass during cycling exercise. As is common practice (Richardson *et al.*, 2004; Mortensen *et al.*, 2005), these authors normalized the absolute VO_2 across the leg during cycling by the entire muscle mass of the lower limb, assuming that all muscles of the lower limb were maximally recruited during the exercise, which may not be the case (Lollgen *et al.*, 1980; Green & Patla, 1992). In addition to the, already recognized, hypoxic/hyperoxic studies, which agree with the current study (Roca *et al.*, 1992; Cardus *et al.*, 1998), the concept that mitochondrial O_2 demand, and not O_2 supply, limits VO_{2max} in untrained subjects is further supported by recently published data from Jacobs *et al.* (2013). In this study the

authors documented that the cycle exercise training-induced increase in VO_{2max} of previously sedentary subjects was related to augmented mitochondrial respiratory capacity and not O_2 delivery.

Determinants of VO_{2max} in Untrained and Trained Subjects

This study provides convincing evidence that VO_{2max} in untrained subjects is determined by the capacity of skeletal muscle mitochondria to consume O_2, while, in contrast, VO_{2max} in trained subjects can be attributed to an O_2 supply limitation to the exercising muscle. In terms of limited O_2 demand, although some differences in the mitochondrial quality of trained and untrained subjects have been reported (Jacobs & Lundby, 2013), it seems most likely that O_2 demand limitation among the untrained is the consequence of differences in mitochondrial density rather than a mitochondrial defect. In term of O_2 supply limitation, as exhibited by the trained subjects, it is recognized that the term O_2 supply is rather general and covers multiple steps of the O_2 cascade, including O_2 diffusion across the lungs, O_2 carriage in the blood, delivery of blood born O_2 to active muscle and, finally, the diffusion of O_2 into the muscle, all of which may contribute to the O_2 supply limitation. Although, central limitations, such as pulmonary diffusion and cardiac output, have been reported to limit O_2 supply during maximal exercise (Dempsey *et al.*, 2008; Levine, 2008), the capacity of these factors is unlikely to have been exhausted during KE exercise, a small muscle mass model (Richardson & Saltin, 1998). Therefore, as KE exercise was the main modality of choice in the current study, it appears that peripheral factors like capillary-muscle diffusion (Richardson *et al.*, 1995) or the ability of the vascular system to transport O_2-rich blood to the exercising muscle may contribute to the discrepancy between VO_{2max} and $_{Mito}VO_{2max}$ in the trained

subjects. The potential for a limitation in terms of vascular conductance is of particular interest to our group, as we have recently published evidence that suggests that vasodilation during exercise may be limited by Endothelin-1 to guard against hypotension (Barrett-O'Keefe *et al.*, 2013), but it is not yet known how this is affected by exercise training.

Implications of a Mitochondrial Reserve Capacity

Although mitochondrial respiratory capacity, at maximal KE exercise, in the trained subjects appears to be in excess, it is likely that this reserve capacity has some other function. Indeed, Gollnick and Saltin (1982) postulated that increasing mitochondrial enzyme capacity, even without increasing VO_{2max}, would have profound effects on endurance performance, by increasing fatty acid utilization, and the subsequent sparing of glycogen. Additionally, some have suggested that mitochondrial reserve capacity may be advantageous as this likely distributes the metabolic work demanded by exercise across a broader network of mitochondria. Thus, no single mitochondrion would be required to operate at a maximal rate, allowing the mitochondria to buffer additional stresses that may be encountered (Sansbury *et al.*, 2011).

With this model in mind, when confronted with a metabolic challenge, untrained subjects without a mitochondrial reserve capacity would be more likely to show outward manifestations of this stress (*e.g.*, mitochondrial dysfunction, exercise intolerance, etc.) than exercise-trained subjects who could buffer such an insult with their mitochondrial reserve. In fact, studies examining mitochondrial reserve capacity in animal models and cultured cells, like cardiomyocytes and fibroblasts, have reported that cells with a greater mitochondrial reserve are more resistant to oxidative-stress-induced mitochondrial

dysfunction (Sansbury *et al.*, 2011), defects due to mitochondrial toxins (Fern, 2003), and cell death (Nickens *et al.*, 2013) than cells with little-to-no reserve capacity. As $_{Body}VO_2max$, which was associated with a greater mitochondrial reserve capacity in the current study (Figure 4.4), has been associated with decreased rates of mortality (Laukkanen *et al.*, 2001) and resistance to disease (Koch *et al.*, 2011; Overmyer *et al.*, 2015), it seems likely that some of the protective effects associated with aerobic fitness may be conferred through this excess capacity and would be worthy of further study.

Conclusion

The unique combination of *in vivo* and *in vitro* measures utilized in this study reveal differing factors that determine VO_{2max} in untrained and trained humans and therefore question the premise of symmorphosis in this important process. Specifically, in the untrained, who utilize their entire mitochondrial capacity during maximal KE exercise, there is evidence against symmorphosis, with VO_{2max} being determined by mitochondrial O_2 demand. While, in contrast, trained subjects exhibit an exercise training-induced mitochondrial reserve that results in skeletal muscle VO_{2max}, *in vivo*, being O_2 supply limited, which also fails to fit the postulate of symmorphosis.

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Table 4.1: Characteristics of Untrained and Trained Subjects

	Untrained	Trained	*P*
Subjects (n)	10	10	
Age (years)	25 ± 4	24 ± 2	0.77
BMI (kg/m^2)	25 ± 1	23 ± 1	0.20
$_{Body}VO_{2max}$ (L/min)	2.9 ± 0.2	4.1 ± 0.2*	0.001
$_{Body}VO_{2max}$ (ml/kg//min)	38 ± 2	59± 1	0.001
Cycling WR_{max} (Watts)	243 ± 38	359 ± 63*	0.001
KE exercise WR_{max} (Watts)	45 ± 7	70 ± 5*	0.001
Total Leg Muscle Mass (kg)	6.35 ±0.3	6.46 ±0.3	0.45
Quadriceps Muscle Mass (kg)	1.94 ± 0.1	2.0 ±0.1	0.64
Muscle Fiber Type I (%)	38 ± 7	51 ± 6	0.20
Muscle Fiber Type II (%)	62 ± 7	49 ± 6	0.20
NCAF	3.0 ± 0.5	3.0 ± 0.1	0.45

* Significantly different than untrained. Body Mass Index, BMI; Whole body maximal oxygen consumption assessed on a cycle ergometer, $_{Body}VO_2max$**;** Work rate maximum, WRmax; Knee extensor exercise, KE; Number of Capillaries Around a Fiber, NCAF.

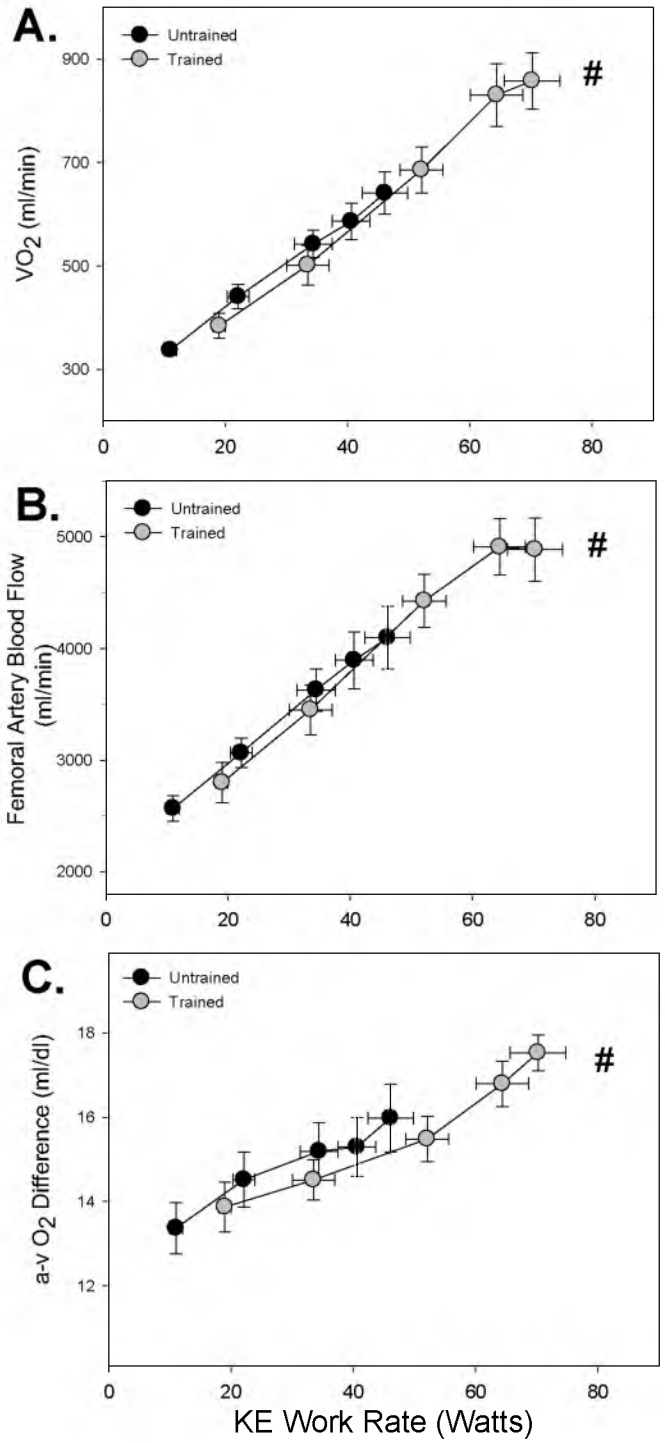

Figure 4.1: **Oxygen consumption, femoral blood flow and arterial-venous oxygen difference during knee extensor (KE) exercise in untrained and trained subjects.** # Significantly different from untrained subjects at maximal KE exercise.

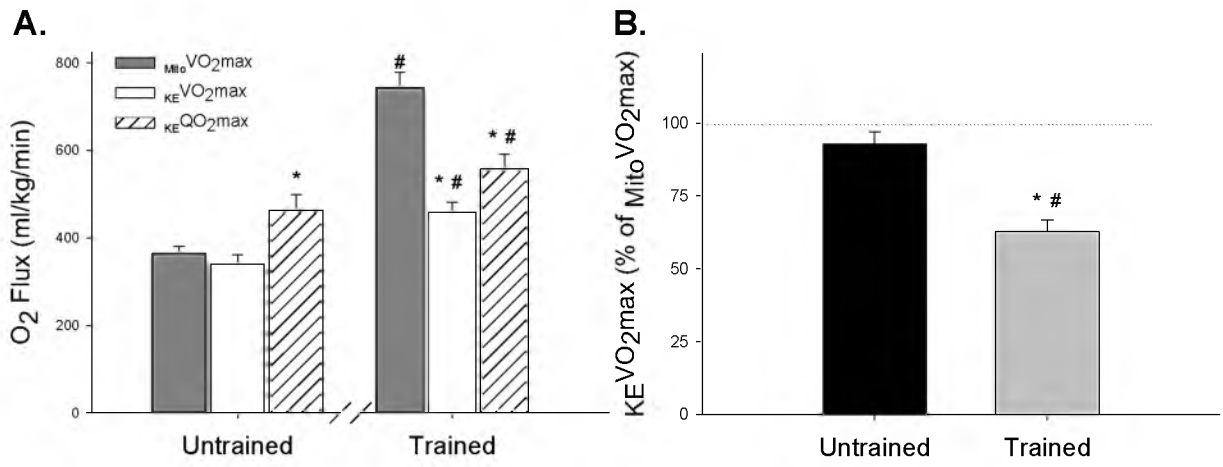

Figure 4.2: Utilization of mitochondrial respiratory capacity during maximal knee extensor (KE) exercise A.) Maximal oxygen consumption ($_{KE}VO_{2max}$) and delivery ($_{KE}QO_{2max}$) during maximal KE exercise in trained and untrained subjects compared to the maximal mitochondrial oxygen consumption ($_{mito}VO_{2max}$) of permeabilized fibers from the vastus lateralis in the same subjects. **B.)** Percent utilization of mitochondrial capacity (% $_{mito}VO_{2max}$) during maximal KE exercise in untrained and endurance-trained subjects. Note, the dotted line in Panel B represents the theoretical point where the full respiratory capacity of the mitochondria are utilized during KE exercise (*i.e.*, 100% $_{Mito}VO_{2max}$) * significantly different from $_{mito}VO_2max$. # Significantly different from untrained subjects.

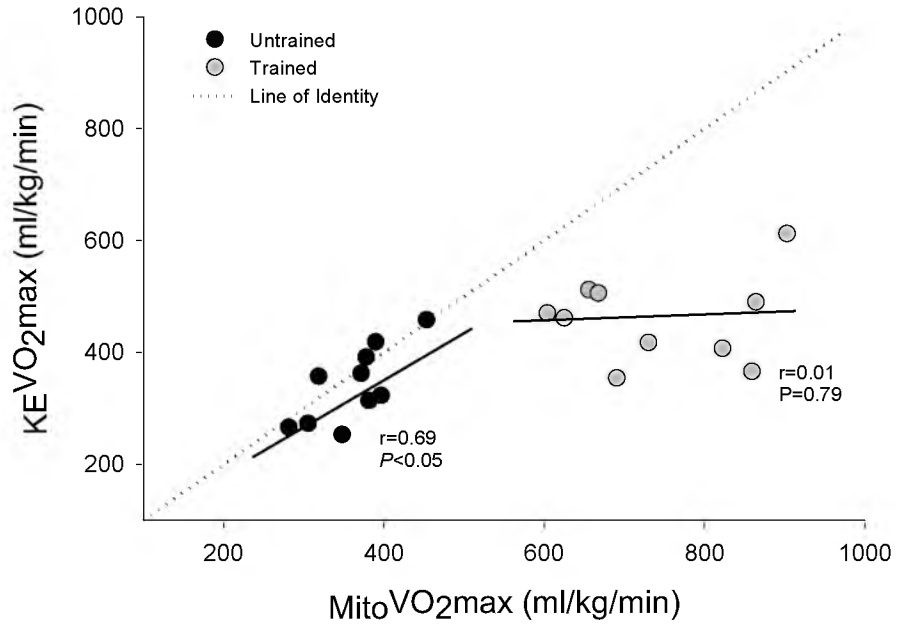

Figure 4.3: Evidence of a relationship between maximal mitochondrial oxygen consumption ($_{Mito}VO_{2max}$) and maximal oxygen consumption during knee extensor (KE) exercise ($_{KE}VO_{2max}$) in untrained, but not trained subjects. The dotted line represents the line of identity (*i.e.*, perfect 1:1 relationship between $_{Mito}VO_{2max}$ and $_{KE}VO_{2max}$).

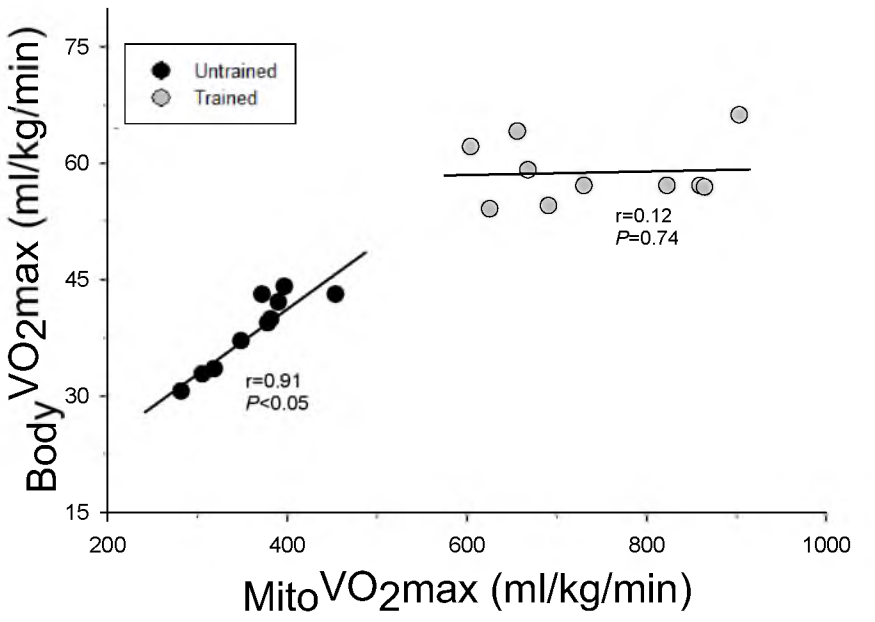

Figure 4.4: Evidence of a relationship between maximal mitochondrial oxygen consumption ($_{Mito}VO_{2max}$) and whole-body oxygen consumption ($_{Body}VO_{2max}$) in untrained, but not trained subjects. $_{Body}VO_{2max}$ assessed by indirect calorimetry during cycle exercise.

CHAPTER 5

CONCLUSION

During dynamic exercise the active muscles require greater quantities of ATP to power muscle contraction and maintain cell function (Richardson, 2003). As much of this ATP is resynthesized by mitochondria in the muscle, the function of the mitochondria is crucial to the health and performance of the muscle. Given the essential role that O_2 plays in mitochondrial resynthesis of ATP, it is equally crucial that a sufficient supply of O_2 is delivered to the mitochondria by the cardiopulmonary system. In view of the critical roles of these processes in maintaining function during exercise, this dissertation sought to elucidate the regulation of skeletal muscle O_2 supply and demand and determine their influence on physical function in the context of health and disease.

In the first study, motivated by the theory that heat generated by an exercising muscle attunes O_2 supply to metabolic demand, we sought to explore the mechanisms by which physiologically-relevant levels of heat inhibit α-adrenergic vasocontraction. Interestingly, we found that α_1-adrenergic vasocontraction was more clearly inhibited by heat than α_2-adrenergic vasocontraction. Additionally, we found that this sympathoinhibitory effect of heat could be prevented by inhibiting the temperature-sensitive, TRPV4 ion channels or by denuding the endothelium. Thus, we concluded that TRPV4 ion channels present in isolated skeletal muscle feed arteries respond to heat, typical of the muscle during exercise, to inhibit α_1-adrenergic vasoconstriction in an endothelium-dependent manner. While these studies were performed in an isolated model, given the potential for feed arteries to regulate blood flow to a muscle (Ives *et al.*, 2012b), it is possible that such mechanisms contribute to functional sympatholysis *in vivo*. In light of the importance of O_2 supply and demand highlighted in the third study of

this dissertation, it seems appropriate and efficient that mechanisms directly linked to the metabolism of the muscle itself calibrate O_2 supply to metabolic demand.

In the second study we sought to determine if qualitative changes in mitochondrial respiratory function (*i.e.*, O_2 demand) contribute to the exercise intolerance exhibited by patients with COPD. Indeed, the muscle of patients with COPD exhibited a lower peak respiration and an altered pattern of respiration compared to healthy, age-matched controls. Notably, the mitochondria of patients with COPD exhibited a diminished capacity for CI-driven respiration and an increased reliance on CII-driven respiration (*i.e.*, reduced CI/CII) compared to controls. Importantly, those patients who exhibited a lower CI/CII fatigued earlier during KE than those who exhibited a greater CI/CII, supporting the notion that this qualitative alteration in mitochondrial respiration contributes to the exercise intolerance typical of patients with COPD. Indeed, as CII-driven respiration demands more O_2 to resynthesize ATP than CI-driven respiration, this altered respiratory profile may partially explain the increased O_2 cost of exercise that is thought to play a role in the exercise intolerance of these patients (Richardson *et al.*, 2004; Medeiros *et al.*, 2014). As comorbidities common to COPD (*e.g.*, pulmonary diffusion limitation and increased work of breathing) often lead to a reduced supply of O_2 to the exercising muscle (Knower *et al.*, 2001; Dempsey *et al.*, 2006), this less-efficient respiratory pattern, which likely exaggerates the O_2 cost of exercise, may be particularly debilitating in patients with COPD.

In the third study, inspired by the theory of symmorphosis, which postulates that the capacity of each step of the O_2 cascade is attuned to the task required at VO_{2max} such that no single step restrains VO_{2max}, we investigated the roles of O_2 supply and O_2

demand in determining VO_{2max} by comparing *in vivo* (skeletal muscle VO_{2max}, direct Fick) and *in vitro* (permeabilized muscle fiber mitochondrial VO_{2max}) measures of respiratory capacity. As endurance training is known to influence both O_2 supply and demand (Gollnick *et al.*, 1973; Levine, 2008) we sought to determine if the limitations of VO_{2max} differed between untrained and endurance-trained subjects. The unique combination of *in vivo* and *in vitro* measures utilized in this study revealed that differing factors limit VO_{2max} in untrained and trained humans and therefore question the premise of symmorphosis in this important process. Specifically, in the untrained, who utilize their entire mitochondrial capacity during maximal KE exercise, there is evidence against symmorphosis, with VO_{2max} being limited by mitochondrial O_2 demand. Conversely, the trained subjects, who also fail to fit the premise of symmorphosis, exhibit an exercise training-induced mitochondrial reserve that results in skeletal muscle VO_{2max}, *in vivo*, being limited by O_2 supply.

The greater understanding of the roles of O_2 supply and O_2 demand in limiting VO_{2max} provided by the third study sheds light on the importance and relevance of the findings of the other two studies. First, the strong influence of O_2 supply on VO_{2max} in the endurance-trained highlights the importance of attuning the delivery of O_2-rich blood to the metabolic demand of exercising muscle through functional sympatholysis. Indeed, it has been documented that inefficient sympatholysis can hinder physical function (Saltin & Mortensen, 2012), and, again, based upon the fact that endurance-trained individuals were strongly limited by O_2 supply, the influence of such sympatholytic systems on performance would likely be magnified in these O_2-limited individuals. Second, the fact that the VO_{2max} of untrained individuals was limited by mitochondrial O_2

demand highlights the significance of the decreased respiratory capacity and function exhibited by patients with COPD. Specifically, it has been suggested that the mitochondria are in excess, even among untrained individuals (Boushel *et al.*, 2011; Boushel & Saltin, 2013), such that any decrement in mitochondrial respiratory function is mitigated by the excess capacity. However, since there was no evidence of a mitochondrial reserve capacity in the untrained subjects, it seems likely that the decreased mitochondrial respiratory function of patients with COPD, who are typically very sedentary, would adversely impact the physical function of these patients. In addition to calling attention to the mitochondrial respiratory function of patients with COPD, these data also highlight the potential importance of appropriate mitochondrial respiratory function in other untrained populations.

In summary, by utilizing a broad range of *in vivo* and *in vitro* methods, this dissertation elucidates the mechanisms that regulate O_2 supply and O_2 demand, and clarifies their impact on exercise performance in healthy young individuals, as well as patients with COPD, thereby highlighting the potential of these factors to serve as potential targets to improve physical performance and health outcomes in both healthy and diseased populations.

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